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(57) Abstract

A promoter derived from an SHH gene, especially the SHH gene of *Arabidopsis thaliana* which is capable of directing expression of a variety of operator genes in both monocotyledonous and dicotyledonous plants. The promoter of the invention may be used for directing expression of pathogen resistance genes to disease or wound sites.

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S-ADENOSYL-L-HOMOCYSTEIN HYDROLASE PROMOTER

The present invention relates to a promoter sequence capable of giving a high level of expression within plant cells. In particular, it relates to a promoter derived from a gene encoding S-adenosyl-Lhomocysteine hydrolase (SHH).

Promoters control the spatial and temporal expression of genes by modulating their level of transcription. Early approaches to genetically engineered crop plants utilised strong constitutive promoters to drive the expression of foreign genes. As strategies in plant biotechnology have become more sophisticated, specific promoters have been used to target transgene expression to a particular tissue or to a particular developmental stage. The promoter of the present invention is especially versatile as it can be used either to give constitutive expression of a gene or to target increased levels of gene expression at sites of wounding or pathogen invasion.

SHH was first described, in rat liver extracts, as the activity responsible for the reversible hydrolysis of S-adenosyl-L-homocysteine (SAH) to adenosine and homocysteine by the cleavage of a thioether bond in SAH [de la Haba, G. and Cantoni, G. L. (1959). *J. Biol. Chem.* **234**, 603-608].

SAH is formed as a direct product of transmethylation reactions involving S-adenosyl-L-methionine (SAM) [Cantoni, G.L. and Scarano, E. (1954). *J. Am. Chem. Soc.* **76**, 4744] and is known to be a potent inhibitor of most SAM mediated methyltransfer reactions. Therefore SAH is converted to homocysteine and adenosine by SHH as shown schematically below:

S-adenosyl-L-methionine (SAM)

$\downarrow\uparrow$ Methyltransferase

Methylated Product + S-adenosyl-L-homocysteine (SAH)

$\downarrow\uparrow$ SHH

Adenosine + L-homocysteine

$\downarrow\uparrow$ N5-methyltetrahydrofolate

Methionine

This pathway for the metabolism of SAH is the only pathway in most species. SHH has been found in all cells tested with the exception of *Escherichia coli* and other related bacteria [Shimzu, S. *et al.* (1984). *Eur. J. Biochem.* **141**, 385-392].

The unique metabolic role of SHH in the removal of SAH and the structural complexity of the enzyme suggest that SHH may have a role in the regulation of the biological utilisation of SAM. SAM serves as a major methyl group donor for numerous highly specific methyltransferase reactions with a large variety of acceptor molecules; for example phenylpropanoid derivatives, cyclic fatty acids, proteins, polysaccharides and nucleic acids [Tabor, C. W. and Tabor, H. (1984). *Adv. Enzymol.* **56**, 251-282]. It should be noted that SAM also has regulatory functions, namely the allosteric stimulation of threonine synthase. In plants, SHH has been studied primarily in relation to the biosynthesis of various phenylpropanoid derivatives.

Enzymes affecting the intracellular levels of SAH are important in the study of plant methylation reactions because it has been demonstrated that many methyltransferases are inhibited by SAH [Deguchi, T. and Barchos, J. (1971). *J. Biol. Chem.* **246**, 3175-3181]. For example, an enzyme catalysing the methylation of caffeic acid was purified from spinach-beet leaves and found to be potently inhibited by SAH [Poulton, J. E. and Butt, V. S. (1976). *Arch. of Biochem. Biophys.* **172**, 135-142]. Other metabolic pathways of the plant which involve transmethylation are the production of lignin and suberin, which are both derived from phenylalanine, through a series of reactions. These reactions include the methylation of caffeic acid into ferulic acid and also the methylation of s-hydroxyferulic acid into sinapic acid. Both these methylation reactions require SAM and hence produce SAH as a byproduct which needs to be removed by SHH to allow further transmethylation.

Once SHH had been isolated, many factors were calculated, such as the enzyme's pH optimum of 8.5, with a 50% activity between pH 6.5-10. Due to the Km value found for the substrate, L-homocysteine, the synthesis of SAH proceeds *in vivo* at a significant rate only when L-homocysteine is accumulated [Poulton, J. E. and Butt, V. S.(1976). *Arch. of Biochem. Biophys.* **172**, 135-142].

In vivo, the adenosine produced by the hydrolysis of SAH is not deaminated but is converted to ADP by the successive action of adenosine kinase and adenylate kinase, both of which enzymes have been demonstrated in spinach-beet leaves. If L-homocysteine

accumulates, it causes inhibition of SHH activity and therefore *in vivo*, L-homocysteine appears to be methylated by N5-methyltetrahydrofolate to methionine. Indeed, this reaction has been demonstrated in pea seedling extracts and spinach and barley leaves. Unlike all animal SHH enzymes, plant SHH is not inhibited by adenosine but is instead stabilised by low concentrations [Jakubowski, H. and Guranowski, A. (1981). *Biochem.* **20**, 6877-6881].

The kinetic evidence shows that SHH is a sensitive regulator of SAH utilisation, its activity depending not only upon favourable concentrations of metabolites in relation to equilibrium conditions but also upon the levels of SAM, adenosine and L-homocysteine maintained within the system. These in turn will act as feed back inhibitors or activators to determine the rate of methylation reactions which are sensitive to the levels of SAH [Poulton, J. E. and Butt, V. S. (1976). *Arch. of Biochem. Biophys.* **172**, 135-142].

As previously mentioned SHH has been found in all organisms tested except *E. coli* and some related species where a two step enzymatic process hydrolyses SAH into adenosine and L-homocysteine. So far the following SHH cDNAs have been isolated and published:-

Rat [Ogawa, H. *et al.* (1987). *Proc. Natl. Acad. Sci. USA.* **84**, 719-723],

Dictostelium discoideum [Kasir, J. *et al.* (1988). *Biochem. Biophys. Res. Commun.* **153**, 359-364]

Human [Coulter-Karis, D. E. and Hershfield, M. S. (1989). *Ann. Hum. Genet.* **53**, 169-175]

Caenorhabditis elegans [Prasad. S. S. *et al.* (1991). EMBL database Accession No. M64306]

Leishmania donovani [Henderson, D. M. and Ullman, B. (1992). EMBL database Accession No. M76556]

Petroselinum crispum [Kawalleck, P. *et al.* (1992). *Proc. Natl. Acad. Sci. USA.* **89**, 4713-4717]

Rhodobacter capsulatus [Sganga, M. W. *et al.* (1992). *Proc. Natl. Acad. Sci. USA.* **89**, 6328-6332]

The high level of homology between SHHs of evolutionary divergent species was highlighted further following isolation of SHH from the rat, from *Dictostelium discoideum*, from the purple non-sulphur photosynthetic bacterium *Rhodobacter capsulatus* and then from parsley (*Petroselinum crispum*). The bacterial SHH shows a remarkable degree of amino acid sequence homology, approximately 65% identity and 77% similarity to the previously isolated

SHHs from rat, *D. discoideum*, human and *C. elegans*. This is one of the highest levels of sequence conservation ever reported between proteins having a similar function in prokaryotes and humans. Similarly, SHH cDNA from parsley is 64% identical to rat cDNA and there is 79% similarity at the amino acid level. The lack of sequence divergence between species may suggest a stringent requirement for SHH to retain its primary structure for function.

Both the *R.capsulatus* and the parsley amino acid sequences have an additional amino acid motif in comparison to the rat, *D.discoideum*, human, *C.elegans* and *L. donovani* sequences. *R.capsulatus* has an additional 36 amino acid region whereas parsley has an additional 41 amino acids. These two additional stretches are found in the same position in the predicted protein sequence, approximately one-third of the distance from the amino terminus. (see Figure 3) although they do not show significant homology.

The present inventors have now isolated SHH from various other plant sources. The first of these was *Asparagus officinalis* and the nucleotide sequence and deduced amino acid sequences for this (SEQ ID NO 1 and SEQ ID NO 2) together with the positions of the restriction sites are shown in Figure 1.

Asparagus SHH also contains the extra stretch of residues earlier found in the other photosynthetic species, parsley and *R. capsulatus* and not in SHH cDNAs from non-photosynthetic species. This 41 amino acid stretch, from amino acids 150 to 190 is as well conserved between the dicotyledon species parsley and the monocotyledon species asparagus as is the rest of the sequence although it is not similar to the 36 amino acid stretch from *R. capsulatus*. This is illustrated in Figures 2 and 3.

Following this, SHH cDNAs were also isolated from other species and one of the species selected was *Arabidopsis thaliana*. The promoter derived from the SHH gene from *A. thaliana* has proved to be particularly useful as it directs a high level of expression of a variety of genes, exemplifed by the reporter genes glucuronidase (GUS) and luciferase (LUC). Promoters from the SHH genes of other species may also be isolated using the same techniques and may also be expected to have useful and advantageous effects.

Therefore, in a first aspect of the present invention, there is provided a promoter derived from an SHH gene.

It is preferred that the SHH gene is that derived from *A. thaliana*.

The pr moter has several useful properties and, in particular, because of the uniformity of the SHH gene over different species, it is capable of directing the expression of a wide variety of effect genes in plants, particularly crop plants such as *Arabidopsis*, tobacco, oil seed rape, potato, tomato, banana, wheat and maize.

The sequence of the *Arabidopsis* promoter (SEQ ID NO 3) is shown in Figure 5 and thus in a second aspect of the invention, there is provided a promoter having the sequence of SEQ ID NO 3 or a sequence of at least 70% homology thereto.

It is preferred that the sequence of the promoter has not less than 80% homology, and, more preferably 90% homology to SEQ ID NO 3.

Since transmethylation reactions are important components of the biosynthetic machinery in most plant cells, the SHH will be expressed in cells throughout the plant. The promoter derived from the SHH will therefore provide a useful control mechanism for expression of any effect gene in a constitutive manner. The effect gene may be an SHH gene but will more usually be an introduced gene. Examples of introduced effect genes which may be linked to the promoter of the present invention include selectable markers such as *Npt*II, the kanomycin resistance gene, the phosphinothricin resistance gene or the phosphinothricin acetyl transferase (PAT) gene and others such as the glucuronidase (GUS) and luciferase (LUC) reporter genes.

The predicted increase in transmethylation and concomitant increase in SHH activity following wounding or pathogen invasion means that the SHH gene will also be useful in providing increased levels of expression of introduced genes at sites of wounding and pathogen invasion. In this respect, the SHH promoter will be particularly useful for targeting expression of disease resistance genes, for example genes encoding antifungal proteins such as those described in our earlier patent applications published as WO92/15691, WO92/21699 and WO93/05153. Using the SHH promoter, these antifungal proteins can be targeted to wound sites to prevent fungal invasion or to sites of infection to prevent further spread of the pathogen. The combined constitutive and wound/pathogen induced expression will thus provide a powerful mechanism for the prevention of disease using introduced genes.

In order to direct expression, the promoter and its associated effect gene must, of course be incorporated into a vector and therefore, in a further aspect of the invention there is provided a vector comprising the promoter of the present invention linked to an effect gene.

For expression in dicotyledonous plants binary agrobacterium vectors are particularly suitable whereas for monocotyledonous plants direct DNA delivery vectors are preferred.

As already mentioned above, the sequence of the SHH gene is conserved to a remarkable extent between species. The promoter of the present invention can therefore be used to direct expression in almost any plant species, whether monocotyledonous or dicotyledonous. It is of particular use in crop species such as wheat, maize, oil seed rape, potato, tomato, banana and tobacco.

Thus in a further aspect of the invention there is provided a plant cell transformed with a vector as described above. Transformation may be acheived by standard techniques.

The invention also provides a genetically transformed plant and parts thereof, such as cells protoplasts and seeds, having stably incorporated into the genome the construct of the present invention. Any plant may be chosen but the crop species listed above are particularly preferred.

As already mentioned, the expression of SHH at disease or wound sites means that the promoter will be of particular use in combating disease when linked to an appropriate effect gene.

Therefore, in a further aspect, the invention provides a method of increasing the resistance of a plant to infection by a pathogenic organism, the method comprising transforming the plant with a vector comprising a promoter according to the first aspect of the invention operably linked to a gene conferring resistance to the pathogenic organism.

Examples of genes conferring resistance to pathogenic organisms include the genes encoding antifungal proteins described in WO92/15691, WO92/21699 and WO93/05153.

The isolation of the promoter of the present invention was achieved as a result of the study of SHH in various plant species. The strategy employed was firstly to isolate the gene encoding asparagus SHH. This confirmed the remarkable degree of sequence identity in the SHH gene between plant species and was used as a basis for the design of polymerase chain reaction (PCR) primers which were used to isolate SHH genes from various other plant species including *Arabidopsis thaliana*. Analysis of the *A. thaliana* SHH gene revealed a 1849 base promoter, the promoter of the present invention, which, further experimentation demonstrated to be a highly versatile promoter capable of directing expression of different genes in a variety of plant species.

The invention will now be further described for the purposes of illustration only with reference to the following examples and to the figures in which:

FIGURE 1 shows the nucleotide and deduced amino acid sequence of asparagus SHH (SEQ ID NOS 1 and 2). In Figure 1, the @ symbols define the positions of the start and finish of the original DB6 clone; the sites indicated were used for the sub-cloning of DB6 and the primers used in PCR experiments are underlined.

FIGURE 2 is a comparison of full length predicted SHH protein from asparagus (Dbf) (SEQ ID NO 2) with SHH protein from parsley (Pcshh), the NAD^{-} binding site has been underlined in all species.

FIGURE 3 is a comparison of SHH predicted amino acid sequence from asparagus (Dbf) with SHH proteins from rat, parsley (Pcshh), *R. capsulatus* (Rcahcy) and *C. elegans* (Cehcg); the NAD^{-} binding site has been underlined in all species and * denotes amino acids conserved in all species.

FIGURE 4A shows the amino acid sequence alignment of cloned PCR products (without the primers) from asparagus (ASP, SEQ ID NO 2), *Arabidopsis* (ARA, SEQ ID NO 4), tobacco (TOB, SEQ ID NO 5), *Brachypodium* (BRA, SEQ ID NO 6) and wheat (WH and WHU, SEQ ID NOS 7 and 8). The * denote amino acids conerved in every sepcies and . denotes conservative amino acid changes.

FIGURE 4B is the same as Figure 4A but with the smaller wheat product removed to highlight sequence conservation between the other five PCR species.

FIGURE 5 shows the SHH promoter sequence from *Arabidopsis thaliana* (SEQ ID NO 3) including the first 30 amino acids used in translational transgene fusions.

FIGURE 6 is a map of the *A. thaliana* gene showing coding sequence, intron and 3' untranslated region. Important restriction sites are also shown.

FIGURE 7 shows the coversion of plasmid pSK AoPR1 FULL LUC *via* pSK AtSHH LUC to pBI101 At SHH Luc and pSK AtSHH-GUS to pBIN 19 AtSHH GUS and pBI101 At SHH Correct to pBIl01 At SHH Wrong.

FIGURE 8 shows a comparison of SHH driven LUC activity in stem sections and wounded leaf in tobacco.

FIGURE 9 shows SHH driven LUC expression in various tissues in tobacco.

FIGURE 10 shows LUC line 11 wounding time course in tobacco.

FIGURE 11 shows LUC line 11; open flower non dehisced in tobacco.

FIGURE 12 shows SHH driven GUS expression during *A. thaliana* seedling development.

FIGURE 13 shows SHH driven GUS expression in various *A. thaliana* tissues.

EXAMPLE 1

Elucidation of cDNA Sequence of Asparagus S-Adenosyl-L-Homocysteine Hydrolase

This research utilised previously constructed cDNA libraries derived from an mRNA population purified from mechanically separated *Asparagus officinalis* cells that had been prepared from asparagus seedlings by grinding in a mortar and pestle [Paul, E. *et al.* (1989), *Plant Science*, 65, 111-117 and Harikrishna, K., *et.al.* (1991), *Journal of Experimental Botany*, 42, 791-797].

Clones were randomly picked from the existing cDNA library made using mRNA extracted from model system cells 1-3 days after mechanical isolation and short stretches of derived sequence data. This data was analysed using Pearson and Lipman searches for homologous known sequences within the EMBL database. A putative asparagus SHH cDNA was identified in this manner and called DB6. This clone was subcloned and the full sequence was derived. The positions of the restriction sites used for this purpose are shown in Figure 1.

The nucleotide sequence itself and the translation of this deduced 1633bp sequence were compared to the published SHH clones (particularly the parsley SHH), which demonstrated that DB6 was not full length, with 11 amino acids being absent from the amino terminus. Therefore existing libraries were rescreened using the DB6 insert as a probe and a full length version isolated (SEQ ID NO 1). Interestingly this version, named DBF, was isolated from a different library from the original clone. DB6 was picked from a day 1-3 library whereas DBF was isolated from a day 1 library. Sequence data revealed DBF to encode the full SHH amino acid sequence of 485 residues (SEQ ID NO 2), with 25bp of 5' untranslated nucleotides, 284bp of 3'-untranslated nucleotides and a polyA+ tail.

Genomic Southern data has shown that the asparagus SHH is probably a member of a small gene family, as was found with the parsley homolog. As with the parsley SHH, the asparagus SHH has been isolated from a model system. However, whereas a fungal elicitor was added to the cultured parsley cells, the asparagus system does not use elicitor treatment

and relies on gene induction due to the mechanical isolation of the cells, and therefore it aims to isolate wound induced genes.

Figures 2 and 3 show the asparagus SHH also contains the extra stretch of residues found in the photosynthetic species parsley and *R.capsulatus* and not in the other cloned SHH cDNAs from non-photosynthetic species. This 41 amino acid stretch, from 150-190 amino acids is as well conserved between the dicotyledon parsley and the monocotyledon asparagus as is the rest of the amino acid sequence, unlike the 36 residue stretch of *R.capsulatus*.

EXAMPLE 2

Isolation of SHH Genes from Other Plant Species and Demonstration of Sequence Conservation

To enable further studies as to the significance of this 'extra' region in photosynthetic organisms amino acid sequence of SHH, PCR (Polymerase Chain Reaction) primers were designed to either side of the 41 amino acid stretch common to parsley and asparagus SHH. The primers designed were the following and are shown in context of the SHH cDNA in Figure 1:-

PCR-1 (SEQ ID NO 10)

5' GCGTCTAGATGCAACATACTTCTCCAACCTAGGA 3'

PCR-2 (SEQ ID NO 11)

5' GCGTCTAGATTAGTCAAACTTGCTCTTGGTAGAC 3'

It was expected that a PCR product of 482bp would be produced in control experiments with asparagus genomic DNA as the template, unless an intron existed between the primer annealing sites in the genomic gene. The possibility of introns between the primer binding sites was ruled out following a PCR experiment showing that the expected 482bp product was obtained. Of this 482bp product, 63bp consist of primer sequence (31bp + 32bp). The first 9bp of each primer, at the 5' end, were designed with an *Xba*I site to facilitate cloning of PCR products.

These PCR primers were used to try and amplify a segment of the SHH gene from several plant species whose DNA was available within the laboratory. For all species tested, similar sized products were obtained. When these products were hybridised to the asparagus SHH cDNA probe good hybridisation was observed. SHH PCR products were amplified from *Arabidopsis*, *Asparagus* (as a control), Tobacco, *Brachypodium* and Wheat. A single 480bp PCR product was produced from the *Arabidopsis*, *Asparagus* and *Brachypodium* experiments; whereas wheat and tobacco both produced further products of 350bp and 700bp respectively, in addition to the predicted size product. In all cases a product of the predicted size was found. The second tobacco product of 700bp was later proved to be this size due to multimers of PCR-2 primer sequence on one end, as a result of ligation or PCR error.

The other wheat product was smaller than predicted (350bp) and when it was cloned and sequenced it was revealed why this was the case. Initial attempts to clone the PCR products into pBluescript (Trade Mark) using the *Xba*I site within the primers failed except for the control product from asparagus. Therefore a commercial vector available specifically for the cloning of PCR products was used, this vector is called PCRII. The vector utilises the fact that Taq polymerase used in PCR will add single deoxyadenosines to the 3'-end of all duplex molecules, therefore eliminating the need for restriction sites within the primers. All the PCR products from each species mentioned were cloned in this manner and then sequenced. This sequence data revealed why the initial attempts at cloning into pBluescript had failed. During the PCR reaction, for an unknown reason, the whole primer had not always been replicated at its 5'-end, causing the recognition site of *Xba*I not to be present in the final product. In most cases one primer had the site while the other did not.

All the clones were sequenced and multiple line-ups performed as can be seen in Figure 4 which compares the deduced amino acid sequences for asparagus (SEQ ID NO 1), *A. thaliana* (SEQ ID NO 4), tobacco (SEQ ID NO 5), *Brachypodium* (SEQ ID NO 6) and the two wheat products (SEQ ID NOS 7 and 8). The smaller of the two wheat products proved to be more closely related to the nonphotosynthetic cDNAs isolated, in that it did not contain the extra stretch of 41 amino acids found in parsley and asparagus. The validity of this product needs to be checked as it may have arisen through contamination. Computer analysis has already proven this not to be the same as the Human SHH, previously cloned. However as

a 480bp wheat product was also cloned this could enhance the argument that SHH genes exist as small gene families encoding enzymes with differing biological/physiological roles.

In summary these data shows the SHH gene sequences to be highly conserved across the plant kingdom for the following reasons; firstly, the PCR primers facilitated the successful amplification of the SHH sequence from every tested plant species and secondly, the actual nucleotide and predicted amino acid sequence of this region shows how conserved the SHH gene is between plant species spanning the monocotyledon/dicotyledon classification. (See Figure 4).

Thus it has been shown that the SHH amino acid sequence is highly conserved between a diverse range of plant species.

EXAMPLE 3

Demonstration of the role of SHH in Transmethylation Reactions

Molecular and Biochemical Characterisation.

It was predicted that an accumulation of SAH would inhibit the SAM mediated caffeic acid-O-methyltransferase reaction.

If, as suggested, SHH has a central role in allowing the transmethylation reactions of several metabolic pathways to occur unhindered it must be present and active in specific regions of the plant at specific developmental periods. Therefore the well studied lignification process occurring in the stems of maturing tobacco where two well characterised transmethylation reactions occur in the biosynthesis of lignin precursors would confirm the point. Although SHH transcript levels may vary between organs, for example lignifying stems, leaves, roots, pollen etc., it does not necessarily mean that the activity of the enzyme will be altered.

To examine the expression of the SHH gene in a range of tobacco organs, steady state mRNA levels were determined using northern analysis and enzyme assays were used to determine the level of enzyme activity.

Northerns were performed using standard techniques with the tobacco PCR product (Figure 4) or cDNA as a hybridisation probe. Extraction of SHH enzyme and assay of activity were performed as follows:

All extraction steps were performed at 4°C.

1. Homogenise plant tissue (~1g) by grinding in a pestle and mortar with 2v/w extraction buffer [100mM Tris pH8, 10mM Sodium Metabisulphite, 10mM Ascorbic Acid and 5mM DTT added on day of use], acid washed sand and 0.1g of insoluble PVP.
2. Decant the supernatant and centrifuge at 17000g for 15min. Remove the supernatant, noting the volume and add 0.56g of solid ammonium sulphate per ml. Stir for 30 min.
3. Centrifuge at 17000g for 15min, resuspend the pellet in 2.5 ml of resuspension buffer [100mM Tris pH8 and 5mM DTT added on day of use] and clarify the solution by pulse centrifugation.
4. The extract is then desalted on a Pharmacia PD-10 G-25 column which has been pre-equilibrated with resuspension buffer according to the manufacturer's protocol. The resultant eluate is used in the following assay procedure.
5. Sequentially add the following to a microcentrifuge tube
   a) 10 µl of 100mM DL-Homocysteine
   b) 80 µl of enzyme extract
   c) 10 µl of Adenosine (100 µl of 53mCi/mmol ^{14}C-adenosine and 100 µl of 20mM adenosine)
6. Mix and incubate at 300C for 30min.
7. Stop the reaction by adding 10 µl of 50% TCA and stand on ice for 10min.
8. Centrifuge and apply 20 µl to a 1.5cm wide strip on a silica TLC plate containing fluorescent indicator (F254, Merck). Develop the plate for a distance of 10cm in butan-1-ol+acetic acid+water (12:3:5).
9. After allowing the plate to dry, visualise the SAH product with a UV lamp at 254nm. Cut out these areas and elute the silica from the plate with 0.5ml methanol before scintillation counting.

Northern analysis showed the SHH transcript to be detected at very low levels in most tissues tested. SHH enzyme assays demonstrated that transcript levels and enzyme activity levels do correlate strongly. Inducible SHH enzyme activity was found in wounded tissue from asparagus, tobacco and *Arabidopsis* when compared with SHH enzyme activity in unwounded tissue. The products of the enzyme assay were separated on a TLC plate

according to Poulton and Butt (*Archives of Biochemistry and Biophysics* **172**, 135-142, 1976) and both ^{14}C labelled adenosine and S-adenosyl homocysteine were detected. The rf values of both ^{14}C labelled compounds compared favourably with those obtained for unlabelled sources that were run on the plates simultaneously and detected by UV fluorescence. In the absence of homocysteine or enzyme preparation, no fluorescent products were observed with the same rf values as unlabelled SAH. These data demonstrate that ^{14}C labelled SAH was derived from the catalytic conversion of ^{14}C labelled adenosine and homocysteine by the SAH enzyme present in the plant preparations.

EXAMPLE 4

Isolation of a SHH gene from *Arabidopsis thaliana*

The PCR fragment of the SHH gene from *Arabidopsis* was used to screen an *Arabidopsis* genomic library for the corresponding gene using standard techniques. Positive clones arising from the screen were analysed and the SHH gene sequenced from a candidate clone containing the gene and its promoter control regions. The DNA sequence of the promoter is shown in Figure 5 and the DNA and deduced amino acid sequence of the coding region in Figure 6.

EXAMPLE 5

SHH gene down-regulation and over-expression studies

The *Arabidopsis* gene sequence described above was used in a series of experiments to modulate SHH gene activity either by down-regulation using antisense or partial sense constructs or by over-expression using the full coding sequence thus reducing the increasing SHH enzyme activity respectively. Effects in specific plant organs or at particular sites of metabolism may be achieved through use of appropriate gene promoters; for example, the lignification process may be modified by using a gene promoter isolated from a gene specific to lignifying tissues such as cinnamoyl:CoA reductase or cinnamyl alcohol dehydrogenase. Alternatively, specific organs may be targeted such as the anthers using the *Arabidopsis* A9 or APG promoters or pollen using the maize ZM13 promoter. Furthermore gene activity could be modified at sites of pathogen attack or wounding through use of wound promoter e.g. AoPRI from asparagus. Finally, SHH enzyme activities may be modified throughout the plant by using a promoter expressed in most plant tissues e.g. CaMV 35S.

EXAMPLE 6

Analysis of *Arabidopsis* SHH promoter activity

The promoter isolated from the *Arabidopsis* SHH gene has been tested in transgenic tobacco plants and in *A. thaliana* to establish its pattern of expression. As shown below this promoter has high level expression in all organs analysed and an additional activity which is induced following wounding. It therefore has utility as a constitutive promoter for expression of selectable markers for *in vitro* selection of transformants or for high level expression in mature plants. Furthermore, the wound induced activity may be used for directing gene products (e.g. antifungal proteins) to sites of wounding or pathogen invasion.

Construction of the SHH promoter - reporter gene were undertaken as follows:

1. **Transcriptional fusions between the SHH promoter and the luciferase (LUC) reporter gene.**

The following construct is based on pSK AoPR1-LUC as described previously (Warner *et al*. The Plant Journal 6:31-43,1994). This construct (Figure 7) was digested with *Nco*I and *Xho*I to remove the AoPRI promoter. Using these sites the *Arabidopsis* SHH promoter was ligated into the plasmid in front of Luc *via* an *Nco*I site to create pSK AtSHH-LUC (Figure 7), a cloning intermediate.

The binary vector pBI01 AoPR1-LUC (Warner *et al*., 1994) was disgested with *Bam*HI and *Sal*I to remove the AoPRI-LUC cassette and the *XhoI/Bam*HI-digested SHH promoter-LUC reporter cassette from pSK AtSHH-LUC (Figure 7) was ligated into the plasmid to create pBI01 AtSHH-LUC (Figure 7/2).

2. **Transcriptional fusions between the SHH promoter and the glucuronidase (GUS) reporter gene.**

Similar SHH promoter-reporter cassettes were constructed utilising the GUS reporter in place of the LUC reporter. This facilitated direct comparisons between the two reporters under the control of the same *Arabidopsis* SHH promoter.

Initially a pSK-derived plasmid containing a NOS terminator behind the GUS gene containing an *Nco*I site at the initiating methionine codon was digested with *NcoI/Xho*I. The *Arabidopsis* SHH promoter was similarly digested and ligated into the vector to create pSK AtSHH-GUS (Figure 7/3). The *XhoI/Bam*HI fragment of this plasmid was then cloned into

the *Bam*HI/*Sal*I sites of BIN19 (Bevan, M. (1984), *Nucleic Acids Research*, **12**, 8711-8721) to create a binary plasmid pBIN19 AtSHH-GUS (Figure 7/3).

3. **Translational fusions between the SHH promoter and the glucuronidase (GUS) reporter gene.**

A simple one step cloning process allowed a further GUS fusion to be made using pBI01. From sequence data it was predicted that a fusion to be made using pBI01 would generate an active transitional fusion between the *Arabidopsis* SHH promoter and GUS with the first 30 amino acids of the GUS fusion encoded by the SHH gene. This construct was made by ligating the 1949 bp *Xho*I fragment of the SHH promoter into the *Sal*I site of pBI01. The resultant clone was named pBI01.1 in the opposite orientation (i.e. in the anti-sense orientation) creating pBI01 AtSHH Wrong (Figure 7/4). This construct (Figure 7/4) was used as a negative control in expression studies.

REPORTER GENE ASSAYS

GUS activity was determined using standard techniques (Jefferson). LUC assays were performed essentially as in Ow *et al*. *Science*, **234**, 856-859, 1986 with modifications described by Warner *et al*., 1994.

Figures 8 -11 show luciferase activity data expressed as light units/μg total protein for one representative transgenic tobacco line. Identical reporter expression patterns were observed in several other SHH promoter-LUC and SHH promoter-GUS tobacco transgenic lines.

Similar patterns of reporter gene expression were also observed within transgenic *A. thaliana*, as demonstrated in Figures 12 and 13. These *A. thaliana* transgenics represent T3 homozygous lines containing a single copy T-DNA. Fluorometric assays of GUS activity within leaves of several fo these lines prove that the exprssion due to the SHH promoter occurs at levels similar to or greater than CaMV35s-driven GUS levels in similar transformants. Of seventy-one individual transformed lines harbouring the pBI121 [Jefferson *et al* (1987) *EMBO J.*, **6**, 3901-3907], the highest activity within leaves was found to be 12040 pmol MU/min/mg, with an average between 2000 and 3000 pmol MU/min/mg [Clarke *et al*, (1992) *Plant Mol. Biol. Reporter*, **10**, 178-189]. Of the five chosen SHH-GUS *A. thaliana* homozygous T3 lines, the expression within leaves varies from 20984 pmol

MU/min/mg to 4420 pmol MU/min/mg with an average of 13725 pmol MU/min/mg, a greater value than the highest expressing line using pBI121.

Histochemically stained transgenic tobacco tissues supported the expression data for GUS activity in all tissues tested.

These results show that the AtSHH promoter drives reporter gene expression in all tissues tested. The point of interest lies in the respective levels of the expression. AtSHH promoter reporter gene expression levels in transgenic plants were far higher than would be predicted from the levels of endogenous SHH transcript. The results in tobacco may be explainable in terms of aberrant expression driven by the *Arabidopsis* promoter in the tobacco host plant due to incorrect transcription factors recognising the introduced promoter but the increased levels of expression in *A. thaliana* suggest that this is not the case. Alternatively, the high levels of reporter gene activity could be a result stabilisation or high levels of translation of the reporter gene transcript affected by the *Arabidopsis* SHH 5' leader sequence present in all constructs made.

The AtSHH promoter, has been demonstrated to cause increased reporter gene expression in tobacco and in *A. thaliana*, and this demonstrates its utility as a high level constitutive promoter.

Furthermore, superimposed on the constitutive expression pattern of the AtSHH promoter is a 2.5-fold increase in expression at wound sites which can be clearly seen in Figure 10.

EXAMPLE 7

To establish utility of the *Arabidopsis* SHH promoter in directing expression of an ATP gene and providing resistance to a fungal pathogen, the 1760 bp promoter fragment from pSKAt SHH-GUS was amplified by PCR using the primers to change the 5' *Xho*I site to *Hind*III and the *Nco*I site at the ATG start codon to *Xho*I. The resulting fragment was cloned directly into a pMJB1 vector as a partial *Hind*III-*Xho*I fragment such that the promoter is placed upstream of the ATP gene. An omega translational enhancer from tobacco mosaic virus, located between the SHH promoter and the ATP gene is included to increase the level of gene expression. In this example, the ATP gene is Dm-AMP1 obtained from seeds of *Dahlia merckii*. The resulting construct was introduced into oilseed rape using standard

Agrobacterium-mediated transformation techniques. Transformed plants wre screened for expression of the Dm-AMP1 gene using western blotting techniques and expressing lines advanced into detached leaf disease assays with the oil seed rape pathogen *Phoma lingam* (Gretenkort and Ingram (1993), *J. Phytopathology*, 137, 89-104). Introduction of the Dm-AMP1 gene and experession by the SHH promoter resulted in increased resistance to infection by *Phoma lingam*. These observations parallel those obtained when expression of the Dm-AMP1 gene is controlled by a well-known constitutive promoter, 35S, from cauliflower mosaic virus, exemplifying the utility of the SHH promoter in this application.

CLAIMS

1. A promoter derived from an SHH gene.

2. A promoter as claimed in claim 1 derived from the SHH gene of *A. thaliana*.

3. A promoter having the nucleotide sequence of SEQ ID NO 3.

4. A promoter having a nucleotide sequence which is at least 70% homologous to SEQ ID NO 3.

5. DNA comprising a promoter as claimed in any one of claims 1 to 4, operably linked to an effect gene.

6. DNA as claimed in claim 5 wherein the effect gene is a gene encoding SHH, an antifungal protein, a selectable marker such as *Npt*II, the kanomycin resistance gene, the phosphinothricin resistance gene or the phosphinothricin acetyl transferase (PAT) gene, the glucuronidase (GUS) reporter gene or the luciferase (LUC) reporter gene.

7. A vector comprising DNA as claimed in claim 5 or claim 6.

8. A vector as claimed in claim 7 which is a binary agrobacterium vectors or a direct DNA delivery vector.

9. A plant cell transformed with a vector as claimed in claim 7.

10. A genetically transformed plant or part thereof, such as a cell, protoplast or seed, having stably incorporated into the genome the DNA as claimed in claim 5 or claim 6.

11. A plant cell or genetically transformed plant, wherein the plant is wheat, maize, oil seed rape, potato, tomato, banana or tobacco.

12. A method of increasing the resistance of a plant to infection by a pathogenic organism, the method comprising transforming the plant with a vector comprising a promoter according to any one of claims 1 to 4 operably linked to a gene conferring resistance to the pathogenic organism.

13. A method as claimed in claim 13 wherein the pathogenic organism is a fungus and the gene encodes an antifungal protein.

Fig.1.

```
                                                              @
        CTCGTTTCAGATCCGATCTGAAGAAATGGCTCTCCTCGTTGAGAAGACTACCTCTGGCCG
     1  ---------+---------+---------+---------+---------+---------+  60
        GAGCAAAGTCTAGGCTAGACTTCTTTACCGAGAGGAGCAACTCTTCTGATGGAGACCGGC

b                                M  A  L  L  V  E  K  T  T  S  G  R  -

        CGAGTACAAGGTCAAGGACATGTCTCAGGCCGACTTCGGCCGCCTCGAGATCGAGCTCGC
    61  ---------+---------+---------+---------+---------+---------+  120
        GCTCATGTTCCAGTTCCTGTACAGAGTCCGGCTGAAGCCGGCGGAGCTCTAGCTCGAGCG

b        E  Y  K  V  K  D  M  S  Q  A  D  F  G  R  L  E  I  E  L  A  -

        TGAGGTCGAGATGCCAGGGCTCATGGCCTGCCGTGCCGAGTTCGGCCCCGCCCAGCCATT
   121  ---------+---------+---------+---------+---------+---------+  180
        ACTCCAGCTCTACGGTCCCGAGTACCGGACGGCACGGCTCAAGCCGGGGCGGGTCGGTAA

b        E  V  E  M  P  G  L  M  A  C  R  A  E  F  G  P  A  Q  P  F  -

                             BamHI
        CAAGGGCGCAAAAATCACTGGATCCCTCCACATGACGATCCAAACTGCCGTCCTCATCGA
   181  ---------+---------+---------+---------+---------+---------+  240
        GTTCCCGCGTTTTTAGTGACCTAGGGAGGTGTACTGCTAGGTTTGACGGCAGGAGTAGCT

b        K  G  A  K  I  T  G  S  L  H  M  T  I  Q  T  A  V  L  I  E  -

                                                   PCR-1 Primer
        AACCCTAACCGCCCTCGGGCCCGAGGTTCGCTGGTGCTCCTGCAACATATTCTCCACCCA
   241  ---------+---------+---------+---------+---------+---------+  300
        TTGGGATTGGCGGGAGCCCGGGCTCCAAGCGACCACGAGGACGTTGTATAAGAGGTGGGT
```

Fig.1 (Cont).

```
b          T  L  T  A  L  G  P  E  V  R  W  C  S  C  N  I  F  S  T  Q  -

           GGACCATGCCGCCGCTGCCATTGCCCGTGACTCCGCCTCCGTCTTCGCCTGGAAGGGTGA
      301  ---------+---------+---------+---------+---------+---------+ 360
           CCTGGTACGGCGGCGACGGTAACGGGCACTGAGGCGGAGGCAGAAGCGGACCTTCCCACT

b          D  H  A  A  A  A  I  A  R  D  S  A  S  V  F  A  W  K  G  E  -

           GACCCTCCAGGAGTACTGGTGGTGCACCGAGCGTGCCCTCGACTGGGGCCCCGGCGGTGG
      361  ---------+---------+---------+---------+---------+---------+ 420
           CTGGGAGGTCCTCATGACCACCACGTGGCTCGCACGGGAGCTGACCCCGGGGCCGCCACC

b          T  L  Q  E  Y  W  W  C  T  E  R  A  L  D  W  G  P  G  G  G  -

           CCCTGACCTCATCGTCGATGACGGCGGCGACACCACTCTCTTGATCCATGAGGGGGTGAA
      421  ---------+---------+---------+---------+---------+---------+ 480
           GGGACTGGAGTAGCAGCTACTGCCGCCGCTGTGGTGAGAGAACTAGGTACTCCCCCACTT

b          P  D  L  I  V  D  D  G  G  D  T  T  L  L  I  H  E  G  V  K  -

           GGCCGAGGAAGAGTACGAGAAGACGGGGAAGATGCCCGATCCGGCGTCTACCGACAATGC
      481  ---------+---------+---------+---------+---------+---------+ 540
           CCGGCTCCTTCTCATGCTCTTCTGCCCCTTCTACGGGCTAGGCCGCAGATGGCTGTTACG

b          A  E  E  E  Y  E  K  T  G  K  M  P  D  P  A  S  T  D  N  A  -
```

Fig.1 (Cont).

```
        TGAGTTCCAGATCGTGCTCACAATCATCAGGGATGGGCTCAAGGTGGACCCCACCAAGTA
    541 ---------+---------+---------+---------+---------+---------+ 600
        ACTCAAGGTCTAGCACGAGTGTTAGTAGTCCCTACCCGAGTTCCACCTGGGGTGGTTCAT

b        E  F  Q  I  V  L  T  I  I  R  D  G  L  K  V  D  P  T  K  Y  -

        CAGGAAGATGAAGGATAGGATTGTCGGTGTGTCGGAGGAGACCACCACCGGGGTCAAGAG
    601 ---------+---------+---------+---------+---------+---------+ 660
        GTCCTTCTACTTCCTATCCTAACAGCCACACAGCCTCCTCTGGTGGTGGCCCCAGTTCTC

b        R  K  M  K  D  R  I  V  G  V  S  E  E  T  T  T  G  V  K  R  -

        GCTTTACCAGATGCAGGCTAACAATTCCCTTCTTTTCCCTGCGATCAATGTCAATGACTC
    661 ---------+---------+---------+---------+---------+---------+ 720
        CGAAATGGTCTACGTCCGATTGTTAAGGGAAGAAAAGGGACGCTAGTTACAGTTACTGAG

b        L  Y  Q  M  Q  A  N  N  S  L  L  F  P  A  I  N  V  N  D  S  -

        CGTCACCAAGAGCAAGTTTGACAATCTGTATGGATGCCGGCACTCTCTTCCCGATGGTCT
    721 ---------+---------+---------+---------+---------+---------+ 780
        GCAGTGGTTCTCGTTCAAACTGTTAGACATACCTACGGCCGTGAGAGAAGGGCTACCAGA
              PCR-2 Primer
b        V  T  K  S  K  F  D  N  L  Y  G  C  R  H  S  L  P  D  G  L  -

        GATGAGGGCCACTGATGTTATGATTGCTGGCAAGGTTGCAGTTGTCTGCGGTTATGGTGA
    781 ---------+---------+---------+---------+---------+---------+ 840
        CTACTCCCGGTGACTACAATACTAACGACCGTTCCAACGTCAACAGACGCCAATACCACT

b        M  R  A  T  D  V  M  I  A  G  K  V  A  V  V  C  G  Y  G  D  -
```

Fig.1 (Cont).

```
                 TGTCGGAGAGGGCTGTGCTGCTGCACTCAAGCAGGCTGGTGCCCGTGTTATTGTGACGGA
            841  ---------+---------+---------+---------+---------+---------+ 900
                 ACAGCCTCTCCCGACACGACGACGTGAGTTCGTCCGACCACGGGCACAATAACACTGCCT

  b               V  G  E  G  C  A  A  A  L  K  Q  A  G  A  R  V  I  V  T  E  -

                                                                          XhoI
                 GATCGACCCCATCTGTGCTCTTCAAGCCCTAATGGAGGGTCTTCAGGTCCTCACCCTCGA
            901  ---------+---------+---------+---------+---------+---------+ 960
                 CTAGCTGGGGTAGACACGAGAAGTTCGGGATTACCTCCCAGAAGTCCAGGAGTGGGAGCT

  b               I  D  P  I  C  A  L  Q  A  L  M  E  G  L  Q  V  L  T  L  E  -

                 GGATGTTGTCTCAGAGGCGGATATCTTTGTTACCACCACCGGTAACAAGGACATCATCAT
            961  ---------+---------+---------+---------+---------+---------+ 1020
                 CCTACAACAGAGTCTCCGCCTATAGAAACAATGGTGGTGGCCATTGTTCCTGTAGTAGTA

  b               D  V  V  S  E  A  D  I  F  V  T  T  T  G  N  K  D  I  I  M  -

                 GCTGGACCACATGAGGAAGATGAAGAACAATGCCATTGTCTGCAACATTGGTCACTTTGA
           1021  ---------+---------+---------+---------+---------+---------+ 1080
                 CGACCTGGTGTACTCCTTCTACTTCTTGTTACGGTAACAGACGTTGTAACCAGTGAAACT

  b               L  D  H  M  R  K  M  K  N  N  A  I  V  C  N  I  G  H  F  D  -

                 CAACGAGATTGACATGCTAGGTTTGGAGACATACCCTGGCATCAAGAGAATCACCATCAA
           1081  ---------+---------+---------+---------+---------+---------+ 1140
                 GTTGCTCTAACTGTACGATCCAAACCTCTGTATGGGACCGTAGTTCTCTTAGTGGTAGTT

  b               N  E  I  D  M  L  G  L  E  T  Y  P  G  I  K  R  I  T  I  K  -
```

Fig.1 (Cont).

```
       GCCCCAGACTGACCGGTGGGTCTTCCCTGAAACCAACACTGGTATAATTGTTCTTGCTGA
  1141 ---------+---------+---------+---------+---------+---------+ 1200
       CGGGGTCTGACTGGCCACCCAGAAGGGACTTTGGTTGTGACCATATTAACAAGAACGACT

b        P  Q  T  D  R  W  V  F  P  E  T  N  T  G  I  I  V  L  A  E  -

       GGGCCGACTCATGAACCTTGGGTGTGCCACTGGTCACCCCAGCTTTGTCATGTCCTGCTC
  1201 ---------+---------+---------+---------+---------+---------+ 1260
       CCCGGCTGAGTACTTGGAACCCACACGGTGACCAGTGGGGTCGAAACAGTACAGGACGAG

b        G  R  L  M  N  L  G  C  A  T  G  H  P  S  F  V  M  S  C  S  -

       CTTCACCAACCAGGTGATTGCTCAGCTAGAGTTGTGGAATGAGAAGGCAAGCGGCAAGTA
  1261 ---------+---------+---------+---------+---------+---------+ 1320
       GAAGTGGTTGGTCCACTAACGAGTCGATCTCAACACCTTACTCTTCCGTTCGCCGTTCAT

b        F  T  N  Q  V  I  A  Q  L  E  L  W  N  E  K  A  S  G  K  Y  -

       TGAGAAGAAGGTTTACGTGCTCCCCAAGCATCTTGATGAGAAAGTAGCAGCGCTTCACTT
  1321 ---------+---------+---------+---------+---------+---------+ 1380
       ACTCTTCTTCCAAATGCACGAGGGGTTCGTAGAACTACTCTTTCATCGTCGCGAAGTGAA

b        E  K  K  V  Y  V  L  P  K  H  L  D  E  K  V  A  A  L  H  L  -

                        HindIII
       GGGCAAGCTCGGAGCCAAGCTTACAAAGCTCAGCCCTTCACAGGCGGACTACATCAGCGT
  1381 ---------+---------+---------+---------+---------+---------+ 1440
       CCCGTTCGAGCCTCGGTTCGAATGTTTCGAGTCGGGAAGTGTCCGCCTGATGTAGTCGCA
```

Fig.1 (Cont).

```
b          G  K  L  G  A  K  L  T  K  L  S  P  S  Q  A  D  Y  I  S  V  -

           CCCCATCGAGGGTCCCTACAAGCCACCTCACTACAGGTACTAGACGCTGTTGTGCCGGGG
     1441  ---------+---------+---------+---------+---------+---------+ 1500
           GGGGTAGCTCCCAGGGATGTTCGGTGGAGTGATGTCCATGATCTGCGACAACACGGCCCC

b          P  I  E  G  P  Y  K  P  P  H  Y  R  Y  *

           AGAGATCATCGCAGCAAGAAAGTATTAAGATTGAAGAAGAGAGTTGTTATGGAGGACATG
     1501  ---------+---------+---------+---------+---------+---------+ 1560
           TCTCTAGTAGCGTCGTTCTTTCATAATTCTAACTTCTTCTCTCAACAATACCTCCTGTAC

           GCTATATTTACTTTATTTCCTACCTATTTCTTGCTGTTTCTCTTTCCGAACTTTTAGACT
     1561  ---------+---------+---------+---------+---------+---------+ 1620
           CGATATAAATGAAATAAAGGATGGATAAAGAACGACAAAGAGAAAGGCTTGAAAATCTGA

           GATCCTCTTCTTCTCTTTGATTTATTACGATATGAATTCTGTTTAAATTTTGCTTATTCT
     1621  ---------+---------+---------+---------+---------+---------+ 1680
           CTAGGAGAAGAAGAGAAACTAAATAATGCTATACTTAAGACAAATTTAAAACGAATAAGA

                     @
           CTAATGATGAGCTAGCAGACATATGTTCTGTGGTAGAATAACGAGGTTTTGAACTTTGTG
     1681  ---------+---------+---------+---------+---------+---------+ 1740
           GATTACTACTCGATCGTCTGTATACAAGACACCATCTTATTGCTCCAAAACTTGAAACAC

           CAAAAAAAAAAAAAAAAAAAAAAAAAA
     1741  ---------+---------+------- 1767
           GTTTTTTTTTTTTTTTTTTTTTTTTTT
```

Fig.2.

```
                1                                                   50
   Dbf.Gap   MALLVEKTTS GREYKVKDMS QADFGRLEIE LAEVEMPGLM ACRAEFGPAQ
 Pcshh.Gap   MALSVEKTAA GREYKVKDMS LADFGRLELE LAEVEMPGLM SCRTEFGPSQ

                51                                                 100
   Dbf.Gap   PFKGAKITGS LHMTIQTAVL IETLTALGPE VRWCSCNIFS TQDHAAAAIA
 Pcshh.Gap   PFKJARITGS LHMTIQTGVL IETLTALGAE VRWCSCNIFS TQDHAAAAIA

                101                                                150
   Dbf.Gap   RDSASVFAWK GETLQEYWWC TERALDWGPG GGPDLIVDDG GDTTLLIHEG
 Pcshh.Gap   RDSCAVFAWK GETLQEYWWC TERALDWGPD GGPDLIVDDG GDATLLIHEG

                151                                                200
   Dbf.Gap   VKAEEEYEKT GKMPDPASTD NAEFQIVLTI IRDGLKVDPT KYRKMKDRIV
 Pcshh.Gap   VKAEEEYKKS GAIPDPASTD NAEFQIVLSI IRDGLKSDPM KYHKMKDRLV
```

Fig.2 (Cont).

```
             201                                                    250
  Dbf.Gap    GVSEETTTGV KRLYQMQANN SLLFPAINVN DSVTKSKFDN LYGCRHSLPD
Pcshh.Gap    GVSEETTTGV KRLYQMQQNG TLLFPAINVN DSVTKSKFCN LYGCRHSLPD

             251                                                    300
  Dbf.Gap    GLMRATDVMI AGKVAVVCGY GDVGEGCAAA LKQAGARVIV TEIDPICALQ
Pcshh.Gap    GLMRATDVMI AGKVALIAGY DGVGKGCAAA MKQAGARVIV TEIDPICALQ

             301                                                    350
  Dbf.Gap    ALMEGLQVLT LEDVVSEADI FVTTTGNKDI IMLDHMRKMK NNAIVCNIGH
Pcshh.Gap    ATMEGLQVLP LEDVVSEVDI FVTTTGNKDI IMVSDMRKMK NNAIVCNIGH

             351                                                    400
  Dbf.Gap    FDNEIDMLGL ETYPGIKRIT IKPQTDRWVF PETNTGIIVL AEGRLMNLGC
Pcshh.Gap    FDNEIDMLGL ETYPGVKRIT IKPQTDRWVF PDTGRGIIIL AEGRLMNLGC

             401                                                    450
  Dbf.Gap    ATGHPSFVMS CSFTNQVIAQ LELWNEKASG KYEKKVYVLP KHLDEKVAAL
Pcshh.Gap    ATGHPSFVMS CSFTNQVIAQ LELWNEKSSG KYEKKVYVLP KHLDEKVAAL

             451                                  485
  Dbf.Gap    HLGKLGAKLT KLSPSQADYI SVPIEGPYKP PHYRY
Pcshh.Gap    HLGKLGAKLT KLSKDQADYI SVPVEGPYKP AHYRY
```

Fig.3.

```
DBF      MALLVEKTTSGREYKVKDMSQADFGRLEIELAEVEMPGLMACRAEFGPAQPFKGAKITGS
PCSHH    MALSVEKTAAGREYKVKDMSLADFGRLELELAEVEMPGLMSCRTEFGPSQPFK-ARITGS
RCAHCY   MA---D-------YIVKDIKLAEFGRKELDIAETEMPGLMACREEFGPSQPLKGARIAGS
RAT      MA---DKLP----YKVADIGLAAWGRKALDIAENEMPGLMRMREMYSASKPLKGARIAGC
CEHHG    MA---QSKPA---YKVADIKLADFGRKEIILAENEMPGLMAMRSKYGPSQPLKGARIAGC
         **   .       * * *.  *  **  . .** ******  *  .....*.* *.*.*

DBF      LHMTIQTAVLIETLTALGPEVRWCSCNIFSTQDHAAAAIARDSASVFAWKGETLQEYWW-
PCSHH    LHMTIQTGVLIETLTALGAEVRWCSCNIFSTQDHAAAAIARDSCAVFAWKGETLQEYWW-
RCAHCY   LHMTIQTAVLIETLKALGADVRWASCNIFSTQDHAAAAIAAGGTPVFAVKGETLEEY-WA
RAT      LHMTVETAVLIETLVALGAEVRWSSCNIFSTQDHAAAAIAKAGIPVFAWKGETDEEYLW-
CEHHG    LHMTIQTAVLIETLTALGAEVQWSSCNIFSTQDHAAAAIAQTGVPVYAWKGETDEEYEW-
         ****..*.****** ***..*.* ****************  . .*.* **** .** *

DBF      CTERALDWGPGGGPDLIVDDGGDTTLLIHEGVKAEEEYEKTGKMPDPASTDNAEFQIVLT
PCSHH    CTERALDWGPDGGPDLIVDDGGDATLLIHEGVKAEEEYKKSGAIPDPASTDNAEFQIVLS
RCAHCY   YTDKIFQFPEGTC-NMILDDGGDATLYILLGARVEAG--ETDLIATPTSEDEV---CLFN
RAT      CIEQTLHFKDG-PLNMILDDGGDLT----------------NLIHTK-------------
CEHHG    CIEQTIVFKDGQPLNMILDDGGDLT----------------NLVHAK-------------
           .. .    .   ..*.***** *                  .
```

Fig.3 (Cont).

```
DBF      IIRDGLKVDPTKYRKMKDRIVGVSEETTTGVKRLYQMQANNSLLFPAINVNDSVTKSKFD
PCSHH    IIRDGLKSDPMKYHKMKDRLVGVSEETTTGVKRLYQMQQNGTLLFPAINVNDSVTKSKFC
RCAHCY   QIKKRMVESPGWFTQQRAAIKGVSEETTTGVHRLYDLHKKGLLPFPAINVNDSVTKSKFD
RAT      ------------HPQLLSGIRGISEETTTGVHNLYKMMANGILKVPAINVNDSVTKSKFD
CEHHG    ------------YPQYLAGIRGLSEETTTGVHNLAKMLAKGDLKVPAINVNDSVTKSKFD
                        .   . *.*********  *  .  .  *  **************

DBF      NLYGCRHSLPDGLMRATDVMIAGKVAVVCGYGDVGEGCAAALKQAGARVIVTEIDPICAL
PCSHH    NLYGCRHSLPDGLMRATDVMIAGKVALIAGYDGVGKGCAAAMKQAGARVIVTEIDPICAL
RCAHCY   NKYGCKESLVDGIRRATDVMMAGKVAVVCGYGDVGKGSAASLRGAGARVKVTEVDPICAL
RAT      NLYGCRESLIDGIKRATDVMIAGKVAVVAGYGDVGKGCAQALRGFGARVIITEIDPINAL
CEHHG    NLYGIRESLPDGIKRATDVMLAGKVAVVAGYGDVGKGSAASLKAFGSRVIVTEIDPINAL
         * **  ..** **. ******.*****.. **..** * *  ...  *.** .**.*** **

DBF      QALMEGLQVLTLEDVVSEADIFVTTTGNKDIIMLDHMRKMKNNAIVCNIGHFDNEIDMLG
PCSHH    QATMEGLQVLPLEDVVSEVDIFVTTTGNKDIIMVSDMRKMKNNAIVCNIGHFDNEIDMLG
RCAHCY   QAAMDGFEVVVLEDVVADADIFITTTGNKDVIRIEHMREMKDMAIVGNIGHFDNEIQVAA
RAT      QAAMEGYEVTTMDEACKEGNIFVTTTGCVDIILGRHFEQMKDDAIVCNIGHFDVEIDVKW
CEHHG    QAAMEGYEVTTLEEAAPKANIIVTTTGCKDIVTGKHFELLPNDAIVCNVGHFDCEIDVKW
         ** *.* .*  ...     .*..****  *..   .   . . *** *.**** **..
```

Fig.3 (Cont).

```
DBF       LETYPGIKRITIKPQTDRWVFPETNTGIIVLAEGRLMNLGCATGHPSFVMSCSFTNQVIA
PCSHH     LETYPGVKRITIKPQTDRWVFPDTGRGIIILAEGRLMNLGCATGHPSFVMSCSFTNQVIA
RCAHCY    LKN-H--KWTNIKDQVDMIEMPSGAR-IILLSEGRLLNLGNATGHPSFVMSASFTNQVLA
RAT       LNE-NAVEKVNIKPQVDRYLLKNGHR-IILLAEGRLVNLGCAMGHPSFVMSNSFTNQVMA
CEHHG     LNT-NATKKDTIKPQVDRYTLKNGRH-VILLAEGRLVNLGCATGHPSFVMSNSFTNQVLA
          *           ** * *           .*.*.****.*** * ******** ******.*

DBF       QLELWNE-KASGKYEKKVYVLPKHLDEKVAALHLGKLGAKLTKLSPSQADYISVPIEGPY
PCSHH     QLELWNE-KSSGKYEKKVYVLPKHLDEKVAALHLGKLGAKLTKLSKDQADYISVPVEGPY
RCAHCY    QIELWTK---GAEYQPGVYILPKSLDEKVARLHLKKIGVKLTTLRPDQAEYIGVTVEGPF
RAT       QIELWTH---PDKYPVGVHFLPKKLDEAVAEAHLGKLNVKLTKLTEKQAQYLGMPINGPF
CEHHG     QVELWTKFGTPQEYKLGLYVLPKTLDEEVAYLHLAQLGVKLTKLSDEQASYLGVPVAGPY
          *.***            *    .  *** *** **  **  ..  *** *   ** *... . **.

DBF       KPPHYRY
PCSHH     KPAHYRY
RCAHCY    KSDHYRY
RAT       KPDHYRY
CEHHG     KPDHYRY
          *. ****
```

Fig.4A.

```
ASP    HAAAAIARDSASVFAWKGETLQEYWWCTERAL-----DWGPGGGPDLIVDDGGDTTLL-I
ARA    HAAAAIARDSAAVFAWKGETLQEYWWCTERAL-----DWGPGGGPDLIVDDGGDATLFRI
TOB    HAAAAIARDSRAVFAWKGETLQEYWWCTERAL-----DWGPGGGPDLIVDDGGDATLL-I
BR     HAAAAIARDSAAVFAWKGETLEEYWWCTERCL-----DWGVGGGPDLIVDDGGDPTLL-I
WHU    RAAAAIARDSASVFAWKGETLQGYWWCTERAL-----DWGPGGGLDLIVDDGGDTTLL-I
WH     QAAAAIAAAGIPVFAWKGETEEEYEWCIEQTILKDGKPW----DANMVLDDGGDLT----
       .******   . .*********.. * ** *. .     *    . ....***** *

ASP    HEGVKAEEEYEKTGKMPDPASTDNAEFQIVLTIIRDGLKVDPTKYRKMKDRIVGVSEETT
ARA    HEGVKAEEIFEKTGQVPDPTSTDNPEFQIVLSIIKEGLQVDPRKYHKMKERLVGVSEETT
TOB    HEGVKAEEEYAKSGKLPDPSSTDNVEFQLVLTIIRDGLKTDPLKYTEMKERLVGVSEETT
BR     HEGVKAEEEFEKSGKIPDPESADNPEFKIVLTIIRDGLKTDARKYRKMKERLVGVSEETT
WHU    HEGVKAEEEYEKTGKMPDPTSTDNAEFQIVLTIIRDGLKVDPTKYRKMKDRIVGVSEETT
WH     -------EILHK-------------------------------KYPQMLERIHGITEETT
              *  *                                **  * .*. *..****

ASP    TGVKRLYQMQANNSLLFPAINVND-
ARA    TGVKRLYQMQENGTLLFPAINVNDS
TOB    TGVKRLYQMQANGTLLFPAINVNDS
BR     TGAKRLYQTQNPGTLLFPAINVNDS
WHU    TGVKRLYQMQANNSLLFLTINVNDS
WH     TGVHRLLDMLKAGTLKVPAINVNNA
       **  ** .     .*   .****.
```

Fig.4B.

```
ASP   HAAAAIARDSASVFAWKGETLQEYWWCTERALDWGPGGGPDLIVDDGGDTTLL-IHEGVK
ARA   HAAAAIARDSAAVFAWKGETLQEYWWCTERALDWGPGGGPDLIVDDGGDATLFRIHEGVK
TU    HAAAAIARDSRAVFAWKGETLQEYWWCTERALDWGPGGGPDLIVDDGGDATLL-IHEGVK
BR    HAAAAIARDSAAVFAWKGETLEEYWWCTERCLDWGVGGGPDLIVDDGGDPTLL-IHEGVK
WHU   RAAAAIARDSASVFAWKGETLQGYWWCTERALDWGPGGGLDLIVDDGGDTTLL-IHEGVK
      .********* .*********. ******* **** *** ********* **. ******

ASP   AEEEYEKTGKMPDPASTDNAEFQIVLTIIRDGLKVDPTKYRKMKDRIVGVSEETTTGVKR
ARA   AEEIFEKTGQVPDPTSTDNPEFQIVLSIIKEGLQVDPRKYHKMKERLVGVSEETTTGVKR
TU    AEEEYAKSGKLPDPSSTDNVEFQLVLTIIRDGLKTDPLKYTEMKERLVGVSEETTTGVKR
BR    AEEEFEKSGKIPDPESADNPEFKIVLTIIRDGLKTDARKYRKMKERLVGVSEETTTGAKR
WHU   AEEEYEKTGKMPDPTSTDNAEFQIVLTIIRDGLKVDPTKYRKMKDRIVGVSEETTTGVKR
      ***  . *.*..*** *.** **..**.**..**. *. **  **.*.********** **

ASP   LYQMQANNSLLFPAINVNDS
ARA   LYQMQENGTLLFPAINVNDS
TU    LYQMQANGTLLFPAINVNDS
BR    LYQTQNPGTLLFPAINVNDS
WHU   LYQMQANNSLLFLTINVNDS
      *** *   .*** .******
```

Fig.5.

```
1   CTCGAGTGTT GACCTTTTCT GGTCGATTGA ATAGAATCGA ATGTCTTAAT
51  CCAGTACCCT CCAGCTTTTA TTTCGTGTAA TTTATTTTCC AAACCTACCA
101 CTACCAGTTT CATAACTCTC GAATAAATTT ATCAAATAGT CTTTTGAGTG
151 CTCAAAGTCT TGGGATAATA AATGGTCAGT GCTATGTATC ACCCGGATGT
201 GAAACATTAT GGGTGGAGAT AGACTATTAT AAATTTATTG AAATATACGA
251 TTGTTACTCG TTTAATAGCA AAAGTAGTAC AATGTATATA GTTTCTATCG
301 AGAACAAGAT CTATTTAAAA TTCGAAAAGT ACATTTAAAA TTCATAAACA
351 TATAAAGATA GTAACATGTT AGATCTGCAT AGTACCACCA AAACAAGAAA
401 AAAGAAACGC ACATCGCCAC ATAATTGCTA TGATTCTCAC TGTCGGCTGC
451 TTTGAAATAT TCGATTCTTT TGGTAAATCA CACAACATAA TATAATTACA
501 ATAAATATAT ATATACTAAA GTATAATTAA TATAATTAAT ACCACATTGT
551 TTAATTCTGT TTTGATCTTT TAAGATCAGT CAGATCCACC GACGTTCCTA
601 CACGCGCAGG TCCAGATCCA AACAGCACAC ACACACACAC AATGCCACTA
651 GTGTAAATGC TTGGTGGCTA TTGCATTTGC ACCTATTGAT ACTCTTTCTT
701 CAAAAACAAG TTATTGTTTT TATTTTCAAC CCAACTTTAA TACGGATTCA
```

Fig.5 (Cont).

```
751   TACTGGGATT TAGGTGTTAA ATCTGATAAT TTAGGTTTGA ATAAGTTGTA
801   TATTTGTTTC TTTGATTAAA AAAAGAACCT ATATATATAC AAAAATAAAT
851   AAAAAGTTCT AGATTTCAAT TTTCCGTATA TAGCGGGTTG AATTGTCTAT
901   TTTAATATGA AAATTGXCGG ATCTTATAAA CAAAATGTTC TGAAATATGT
951   AAAAGGATTT AGCCAAAGTT AACCAAAAAA AAAAAAACAA ACAGAAAAGT
1001  CACATTCACA TGTCGTGGTA GATCTAAGGC ATTAATTTAG AAATATGTCG
1051  TTACAATAAG CGGAGAACAT GGGACGTTTC TCGTGGTCCA ATCAGACGAA
1101  CGAGATCTCA TAAATTAAAT GACTTCAGXC GAGGGAATTC ATGGCAGAAT
1151  GATAATGCAA CTTAAGTGAC TTTAGAGTGA AAATGATACG AGAACAATGC
1201  ATAATCCATA TGACCGTTGA GTGAGTGATA CCATTAGCGC GATACAAGCG
1251  GGACTATAAA CTGATCTAGA TTGTTTTTCT TGGGAAAAAA TGTTACAAAT
1301  TTTAAATATG TAGTTTGAAT TGTTAAACCA AGATTCAACA GAAATATACC
1351  GTAAATAAAC AACAGTTGAT AATAGTCATC GAAAAGATAT CAACTGATTC
1401  TTCACTTGGG CTACTGTGAC GGCCCGTTAG GTTCTCAATA TAAGTCAATA
1451  ACTACGATCT ACGATTCACT GAAACAAATA AAACACAGCC ACGTGTCCAC
```

Fig.5 (Cont).

```
1501  CCTCCCACAT CACCGTCCGA TCTAACCCAC GACAAGCTTA CAACACGGGT
1551  CATACCGGCT CGTGCAGCGT GTTCCGTCAT CCACGGGATT ACAACTTCTA
1601  CCAGATCCAC CAAACCCTCA AAACAATCTG AACCGTTCAT TTCATTTTGA
1651  CCTCATCTAT ATATTCTCTG TCACTCCCCT TTCTCTTCTC CTCGCACACA
1701  CTTCTCTCTC TCTCTCTCTC TGCCTCCTTT CGGATTCAAA TCTCAGATCT
                    Met
1751  AGCTCAACCA TGGCGTTGCT CGTCGAGAAG ACCTCAAGTG GCCGTGAATA
1801  CAAGGTCAAA GACATGTCTC AAGCCGATTT CGGTCGTCTC GAACTCGAG
```

Fig.6.

```
          ATGGCGTTGCTCGTCGAGAAGACCTCAAGTGGCCGTGAATACAAGGTCAAAGACATGTCT
     1760 ---------+---------+---------+---------+---------+---------+ 1819
          TACCGCAACGAGCAGCTCTTCTGGAGTTCACCGGCACTTATGTTCCAGTTTCTGTACAGA

a          G  V  A  R  R  E  D  L  K  W  P  *  I  Q  G  Q  R  H  V  S  -
b         M  A  L  L  V  E  K  T  S  S  G  R  E  Y  K  V  K  D  M  S   -
c          W  R  C  S  S  R  R  P  Q  V  A  V  N  T  R  S  K  T  C  L  -

                                   X
                                   h
                                   o
                                   I
          CAAGCCGATTTCGGTCGTCTCGAACTCGAGCTCGCCGAAGTTGAGATGCCTGGACTCATG
     1820 ---------+---------+---------+---------+---------+---------+ 1879
          GTTCGGCTAAAGCCAGCAGAGCTTGAGCTCGAGCGGCTTCAACTCTACGGACCTGAGTAC

a          S  R  F  R  S  S  R  T  R  A  R  R  S  *  D  A  W  T  H  G  -
b         Q  A  D  F  G  R  L  E  L  E  L  A  E  V  E  M  P  G  L  M   -
c          K  P  I  S  V  V  S  N  S  S  S  P  K  L  R  C  L  D  S  W  -

                       E
                       c
                       o
                       R
                       I
          GCTTGTCGTACCGAATTCGGACCTTCTCAGGCATTCAAAGGCGCTAGAATCACCGGATCT
     1880 ---------+---------+---------+---------+---------+---------+ 1939
          CGAACAGCATGGCTTAAGCCTGGAAGAGTCCGTAAGTTTCCGCGATCTTAGTGGCCTAGA
```

Fig.6 (Cont).

```
a          L  S  Y  R  I  R  T  F  S  G  I  Q  R  R  *  N  H  R  I  S -
b         A  C  R  T  E  F  G  P  S  Q  A  F  K  G  A  R  I  T  G  S  -
c          L  V  V  P  N  S  D  L  L  R  H  S  K  A  L  E  S  P  D  L -

          CTTCACATGACCATCCAAACCGCCGTACTCATCGAAACCCTAACTGCTCTCGGTGCTGAA
     1940 +---------+---------+---------+---------+---------+--------- 1999
          GAAGTGTACTGGTAGGTTTGGCGGCATGAGTAGCTTTGGGATTGACGAGAGCCACGACTT

a          S  H  D  H  P  N  R  R  T  H  R  N  P  N  C  S  R  C  *  S -
b         L  H  M  T  I  Q  T  A  V  L  I  E  T  L  T  A  L  G  A  E  -
c          F  T  *  P  S  K  P  P  Y  S  S  K  P  *  L  L  S  V  L  K -

          GTCAGATGGTGTTCCTGCAACATCTTTTCCACTCAAGACCACGCCGCCGCAGCCATCGCT
     2000 +---------+---------+---------+---------+---------+--------- 2059
          CAGTCTACCACAAGGACGTTGTAGAAAAGGTGAGTTCTGGTGCGGCGGCGTCGGTAGCGA

a          Q  M  V  F  L  Q  H  L  F  H  S  R  P  R  R  R  S  H  R  S -
b         V  R  W  C  S  C  N  I  F  S  T  Q  D  H  A  A  A  A  I  A  -
c          S  D  G  V  P  A  T  S  F  P  L  K  T  T  P  P  Q  P  S  L -

          CGTGACTCCGCCGCTGCTTTCGCCTGGAAAGGTGAGACTCTTCAGGAGTACTGGTGGTGT
     2060 +---------+---------+---------+---------+---------+--------- 2119
          GCACTGAGGCGGCGACGAAAGCGGACCTTTCCACTCTGAGAAGTCCTCATGACCACCACA

a          *  L  R  R  C  F  R  L  E  R  *  D  S  S  G  V  L  V  V  Y -
```

Fig.6 (Cont).

```
b        R D S A A A F A W K G E T L Q E Y W W C -
c        V T P P L L S P G K V R L F R S T G G V -

                   X
                   b
                   a
                   I
         ACCGAGCGTGCTCTAGATTGGGGTCCAGGTGGTGGTCCTGATCTGATTGTTGATGATGGT
    2120 +---------+---------+---------+---------+---------+--------- 2179
         TGGCTCGCACGAGATCTAACCCCAGGTCCACCACCAGGACTAGACTAACAACTACTACCA

a         R A C S R L G S R W W S * S D C * * W W -
b        T E R A L D W G P G G G P D L I V D D G -
c         P S V L * I G V Q V V V L I * L L M M V -

         GGTGACGCTACTCTTTTGATTCATGAGGGTGTTAAAGCTGAGGAGATCTTTGAGAAGACT
    2180 +---------+---------+---------+---------+---------+--------- 2239
         CCACTGCGATGAGAAAACTAAGTACTCCCACAATTTCGACTCCTCTAGAAACTCTTCTGA

a         * R Y S F D S * G C * S * G D L * E D W -
b        G D A T L L I H E G V K A E E I F E K T -
c         V T L L F * F M R V L K L R R S L R R L -

         GGTCAAGTTCCTGATCCTACTTCTACTGATAACCCTGAGTTTCAGATCGTGTTGTCTATT
    2240 +---------+---------+---------+---------+---------+--------- 2299
         CCAGTTCAAGGACTAGGATGAAGATGACTATTGGGACTCAAAGTCTAGCACAACAGATAA

a         S S S * S Y F Y * * P * V S D R V V Y Y -
b        G Q V P D P T S T D N P E F Q I V L S I -
c         V K F L I L L L L I T L S F R S C C L L -
```

Fig.6 (Cont).

```
        ATCAAGGAAGGTCTTCAAGTTGATCCTAAGAAGTACCACAAGATGAAGGAGAGACTTGTT
   2300 +---------+---------+---------+---------+---------+--------- 2359
        TAGTTCCTTCCAGAAGTTCAACTAGGATTCTTCATGGTGTTCTACTTCCTCTCTGAACAA

a        Q  G  R  S  S  S  *  S  *  E  V  P  Q  D  E  G  E  T  C  W -
b       I  K  E  G  L  Q  V  D  P  K  K  Y  H  K  M  K  E  R  L  V  -
c        S  R  K  V  F  K  L  I  L  R  S  T  T  R  *  R  R  D  L  L -

        GGTGTCTCTGAGGAAACTACCACTGGTGTTAAGAGGCTTTACCAGATGCAGCAAAATGGA
   2360 +---------+---------+---------+---------+---------+--------- 2419
        CCACAGAGACTCCTTTGATGGTGACCACAATTCTCCGAAATGGTCTACGTCGTTTTACCT

a        C  L  *  G  N  Y  H  W  C  *  E  A  L  P  D  A  A  K  W  N -
b       G  V  S  E  E  T  T  T  G  V  K  R  L  Y  Q  M  Q  Q  N  G  -
c        V  S  L  R  K  L  P  L  V  L  R  G  F  T  R  C  S  K  M  E -

                                                             intron
        ACTCTTTTGTTCCCTGCCATTAACGTTAACGACTCTGTCACCAAGAGCAAGGTATTGATC
   2420 +---------+---------+---------+---------+---------+--------- 2479
        TGAGAAAACAAGGGACGGTAATTGCAATTGCTGAGACAGTGGTTCTCGTTCCATAACTAG

a        S  F  V  P  C  H  *  R  *  R  L  C  H  Q  E  Q  G  I  D  L -
b       T  L  L  F  P  A  I  N  V  N  D  S  V  T  K  S  K  V  L  I  -
c        L  F  C  S  L  P  L  T  L  T  T  L  S  P  R  A  R  Y  *  S -

        TTCAGATTGTTCTTACTAGTGATGATAATCTGCTAGGTCTTAGCTCTGTAGTTTTGATGC
   2480 +---------+---------+---------+---------+---------+--------- 2539
        AAGTCTAACAAGAATGATCACTACTATTAGACGATCCAGAATCGAGACATCAAAACTACG

a        Q  I  V  L  T  S  D  D  N  L  L  G  L  S  S  V  V  L  M  R -
b       F  R  L  F  L  L  V  M  I  I  C  *  V  L  A  L  *  F  *  C  -
c        S  D  C  S  Y  *  *  *  *  S  A  R  S  *  L  C  S  F  D  A -
```

Fig.6 (Cont).

```
                                          H
                                          i
                                          n
                                          d
                                          I
                                          I
                                          I
       GATAGGCTTAACAACAACATAATCACTAAGTAGAAGCAAGCTTTTCATTGTTGGTTGTTG
  2540 ---------+---------+---------+---------+---------+---------+ 2599
       CTATCCGAATTGTTGTTGTATTAGTGATTCATCTTCGTTCGAAAAGTAACAACCAACAAC

a        * A * Q Q H N H * V E A S F S L L V V E -
b       D R L N N N I I T K * K Q A F H C W L L  -
c        I G L T T T * S L S R S K L F I V G C * -

       AAAGTATTACGATCTAGTAGAGAAAAGTCCTTCCTTGGTTACAGAATTTGCATGAAAAGT
  2600 ---------+---------+---------+---------+---------+---------+ 2659
       TTTCATAATGCTAGATCATCTCTTTTCAGGAAGGAACCAATGTCTTAAACGTACTTTTCA

a        S I T I * * R K V L P W L Q N L H E K S -
b       K V L R S S R E K S F L G Y R I C M K S  -
c        K Y Y D L V E K S P S L V T E F A * K V -

       CAATATTTCTCATGCTTTCTTTGATTTTATATTCAACGTTTTGTTTACACTTATGTGCTG
  2660 ---------+---------+---------+---------+---------+---------+ 2719
       GTTATAAAGAGTACGAAAGAAACTAAAATATAAGTTGCAAAACAAATGTGAATACACGAC

a        I F L M L S L I L Y S T F C L H L C A V -
b       Q Y F S C F L * F Y I Q R F V Y T Y V L  -
c        N I S H A F F D F I F N V L F T L M C C -
```

Fig.6 (Cont).

```
     TTACTGATAAATTCATTTGTTCTTGATTTACAGTTCGACAACTTGTATGGTTCCGGTCAC
2720 +---------+---------+---------+---------+---------+--------- 2779
     AATGACTATTTAAGTAAACAAGAACTAAATGTCAAGCTGTTGAACATACCAAGGCCAGTG

a     T  D  K  F  I  C  S  *  F  T  V  R  Q  L  V  W  F  R  S  L -
b    L  L  I  N  S  F  V  L  D  L  Q  F  D  N  L  Y  G  S  G  H  -
c     Y  *  *  I  H  L  F  L  I  Y  S  S  T  T  C  M  V  P  V  T -

     TCACTCCCTGATGGTCTCATGAGGGCCACTGATGTCATGATCGCTGGAAAGGTTGCTGTT
2780 +---------+---------+---------+---------+---------+--------- 2839
     AGTGAGGGACTACCAGAGTACTCCCGGTGACTACAGTACTAGCGACCTTTCCAACGACAA

a     T  P  *  W  S  H  E  G  H  *  C  H  D  R  W  K  G  C  C  Y -
b    S  L  P  D  G  L  M  R  A  T  D  V  M  I  A  G  K  V  A  V  -
c     H  S  L  M  V  S  *  G  P  L  M  S  *  S  L  E  R  L  L  L -

     ATCTGTGGATATGGTGATGTTGGAAAGGGTTGTGCTGCTGCCATGAAGACTGCTGGTGCT
2840 +---------+---------+---------+---------+---------+--------- 2899
     TAGACACCTATACCACTACAACCTTTCCCAACACGACGACGGTACTTCTGACGACCACGA

a     L  W  I  W  *  C  W  K  G  L  C  C  C  H  E  D  C  W  C  * -
b    I  C  G  Y  G  D  V  G  K  G  C  A  A  A  M  K  T  A  G  A  -
c     S  V  D  M  V  M  L  E  R  V  V  L  L  P  *  R  L  L  V  L -

                                               H
                                               i
                                               n
                                               d
                                               I
                                               I
                                               I
     AGAGTCATTGTGACTGAGATTGATCCCATCTGTGCCCTTCAAGCTTTGATGGAAGGACTT
```

Fig.6 (Cont).

```
         2900 +---------+---------+---------+---------+---------+--------- 2959
              TCTCAGTAACACTGACTCTAACTAGGGTAGACACGGGAAGTTCGAAACTACCTTCCTGAA

a               S  H  C  D  *  D  *  S  H  L  C  P  S  S  F  D  G  R  T  S -
b             R  V  I  V  T  E  I  D  P  I  C  A  L  Q  A  L  M  E  G  L  -
c              E  S  L  *  L  R  L  I  P  S  V  P  F  K  L  *  W  K  D  F  -

                                                      E
                                                      c
                                                      o
                                                      R
                                                      V
              CAGGTTCTTACCCTTGAGGATGTTGTCTCAGAAGCTGATATCTTTGTCACCACCACCGGT
         2960 +---------+---------+---------+---------+---------+--------- 3019
              GTCCAAGAATGGGAACTCCTACAACAGAGTCTTCGACTATAGAAACAGTGGTGGTGGCCA

a               G  S  Y  P  *  G  C  C  L  R  S  *  Y  L  C  H  H  H  R  * -
b             Q  V  L  T  L  E  D  V  V  S  E  A  D  I  F  V  T  T  T  G  -
c              R  F  L  P  L  R  M  L  S  Q  K  L  I  S  L  S  P  P  P  V  -

                                S
                                a
                                l
                                I
              AACAAAGACATCATCATGGTCGACCACATGAGGAAGATGAAGACCAACCCTATTGTGTCA
         3020 +---------+---------+---------+---------+---------+--------- 3079
              TTGTTTCTGTAGTAGTACCAGCTGGTGTACTCCTTCTACTTCTGGTTGGGATAACACAGT

a               Q  R  H  H  H  G  R  P  H  E  E  D  E  D  Q  P  Y  C  V  N -
b             N  K  D  I  I  M  V  D  H  M  R  K  M  K  T  N  P  I  V  S  -
c              T  K  T  S  S  W  S  T  T  *  G  R  *  R  P  T  L  L  C  Q  -
```

Fig.6 (Cont).

```
         ACCATTGGTCACTTTGACAATGAGATTGACATGCCTGGACTTGAGACTTACCCTGGTGTG
    3080 +---------+---------+---------+---------+---------+--------- 3139
         TGGTAACCAGTGAAACTGTTACTCTAACTGTACGGACCTGAACTCTGAATGGGACCACAC

a         H  W  S  L  *  Q  *  D  *  H  A  W  T  *  D  L  P  W  C  E -
b        T  I  G  H  F  D  N  E  I  D  M  P  G  L  E  T  Y  P  G  V  -
c         P  L  V  T  L  T  M  R  L  T  C  L  D  L  R  L  T  L  V  * -

         AAGCGTATCACCATCAAGCCACAGACTGACAGGTGGGTGTTCCCAGAGACCAAGGCTGGA
    3140 +---------+---------+---------+---------+---------+--------- 3199
         TTCGCATAGTGGTAGTTCGGTGTCTGACTGTCCACCCACAAGGGTCTCTGGTTCCGACCT

a         A  Y  H  H  Q  A  T  D  *  Q  V  G  V  P  R  D  Q  G  W  N -
b        K  R  I  T  I  K  P  Q  T  D  R  W  V  F  P  E  T  K  A  G  -
c         S  V  S  P  S  S  H  R  L  T  G  G  C  S  Q  R  P  R  L  E -

         ATCATTGTCTTGGCTGAGGGTCGTCTGATGAACTTGGGTTGTCCCACTGGTCACCCAAGT
    3200 +---------+---------+---------+---------+---------+--------- 3259
         TAGTAACAGAACCGACTCCCAGCAGACTACTTGAACCCAACAGGGTGACCAGTGGGTTCA

a         H  C  L  G  *  G  S  S  D  E  L  G  L  S  H  W  S  P  K  F -
b        I  I  V  L  A  E  G  R  L  M  N  L  G  C  P  T  G  H  P  S  -
c         S  L  S  W  L  R  V  V  *  *  T  W  V  V  P  L  V  T  Q  V -

                                                 X
                                                 h
                                                 o
                                                 I
         TTCGTGATGTCTTGCTCTTTCACCAACCAGGTGATTGCCCAGCTCGAGCTCTGGAACGAG
    3260 +---------+---------+---------+---------+---------+--------- 3319
         AAGCACTACAGAACGAGAAAGTGGTTGGTCCACTAACGGGTCGAGCTCGAGACCTTGCTC
```

Fig.6 (Cont).

```
a       R D V L L F H Q P G D C P A R A L E R E -
b      F V M S C S F T N Q V I A Q L E L W N E  -
c       S * C L A L S P T R * L P S S S S G T R -

       AAAGCAAGCGGAAAGTACGAGAAGAAGGTGTACGTTCTTCCCAAGCATTTGGATGAGAAG
  3320 +---------+---------+---------+---------+---------+--------- 3379
       TTTCGTTCGCCTTTCATGCTCTTCTTCCACATGCAAGAAGGGTTCGTAAACCTACTCTTC

a       S K R K V R E E G V R S S Q A F G * E G -
b      K A S G K Y E K K V Y V L P K H L D E K  -
c       K Q A E S T R R R C T F F P S I W M R R -

                            HindIII

       GTTGCATTACTGCACTTGGGCAAGCTTGGAGCCAGGCTTACAAAGCTGTCAAAGGACCAA
  3380 +---------+---------+---------+---------+---------+--------- 3439
       CAACGTAATGACGTGAACCCGTTCGAACCTCGGTCCGAATGTTTCGACAGTTTCCTGGTT

a       C I T A L G Q A W S Q A Y K A V K G P I -
b      V A L L H L G K L G A R L T K L S K D Q  -
c       L H Y C T W A S L E P G L Q S C Q R T N -
                                                          STDP
       TCTGACTACGTCAGCATTCCAATTGAGGGACCATACAAGCCTCCTCACTACAGGTAC|TGA|
  3440 +---------+---------+---------+---------+---------+--------- 3499
       AGACTGATGCAGTCGTAAGGTTAACTCCCTGGTATGTTCGGAGGAGTGATGTCCATGACT
```

Fig.6 (Cont).

```
a           * L R Q H S N * G T I Q A S S L Q V L R -
b          S D Y V S I P I E G P Y K P P H Y R Y *  -
c           L T T S A F Q L R D H T S L L T T G T E -

                  S
                  a
                  l
                  I
           GAGAGAGAGAGAGTCGACAAAGCGGGTCAGGTTCGGATCTGCTTGTGGTTTTTGGGTTGG
      3500 +---------+---------+---------+---------+---------+--------- 3559
           CTCTCTCTCTCTCAGCTGTTTCGCCCAGTCCAAGCCTAGACGAACACCAAAAACCCAACC

a           E R E S R Q S G S G S D L L V V F G L G -
b          E R E R V D K A G Q V R I C L W F L G W  -
c           R E R E S T K R V R F G S A C G F W V G -

           GTGGTGGGGGGAGAGTCGGGACAGCGTGGAGATGTTGGGTCTTCTTGATGAAGGTGGACC
      3560 +---------+---------+---------+---------+---------+--------- 3619
           CACCACCCCCCTCTCAGCCCTGTCGCACCTCTACAACCCAGAAGAACTACTTCCACCTGG

a           G G G R V G T A W R C W V F L M K V D Q -
b          V V G G E S G Q R G D V G S S * * R W T  -
c           W W G E S R D S V E M L G L L D E G G P -

           AGATTTTCAAGTATTTATTAAGGGTTATTGGGCTTTGGGGGGGTGGGCGCTTGGGTTTCT
      3620 +---------+---------+---------+---------+---------+--------- 3679
           TCTAAAAGTTCATAAATAATTCCCAATAACCCGAAACCCCCCCACCCGCGAACCCAAAGA

a           I F K Y L L R V I G L W G G G R L G F F -
b          R F S S I Y * G L L G F G G V G A W V S  -
c           D F Q V F I K G Y W A L G G W A L G F L -
```

Fig.6 (Cont).

```
          TCCACGCTCCCAGAAAACTTG
     3680 +---------+---------+ 3700
          AGGTGCGAGGGTCTTTTGAAC

a          H  A  P  R  K  L    -
b         S  T  L  P  E  N  L  -
c          P  R  S  Q  K  T    -
```

Enzymes that do cut:

EcoRI EcoRV HindIII SalI XbaI XhoI

Enzymes that do not cut:

BamHI NotI

Fig.7 i.

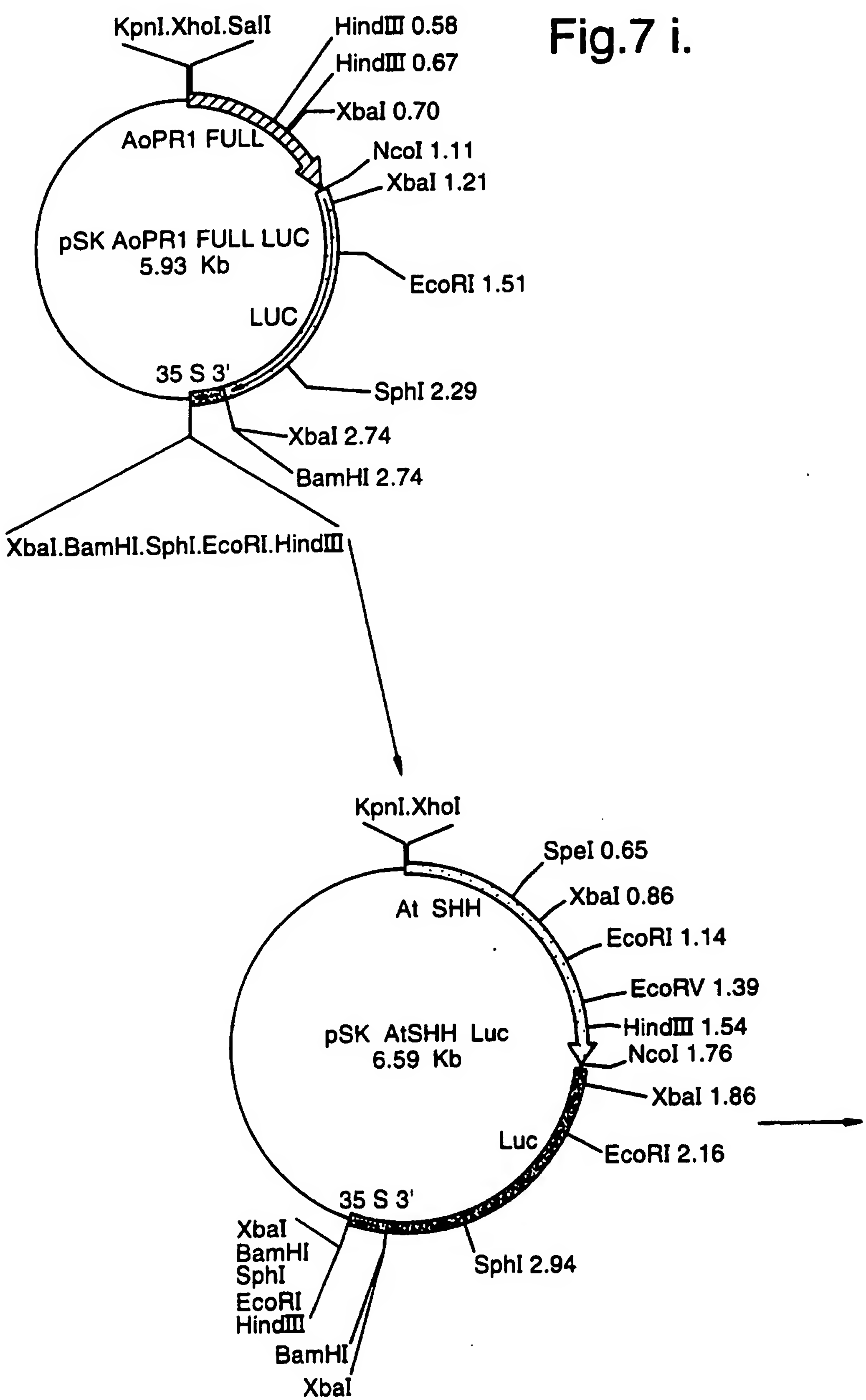

KpnI.XhoI.SalI
HindIII 0.58
HindIII 0.67
XbaI 0.70
AoPR1 FULL
NcoI 1.11
XbaI 1.21
pSK AoPR1 FULL LUC
5.93 Kb
EcoRI 1.51
LUC
35 S 3'
SphI 2.29
XbaI 2.74
BamHI 2.74
XbaI.BamHI.SphI.EcoRI.HindIII
KpnI.XhoI
SpeI 0.65
XbaI 0.86
At SHH
EcoRI 1.14
EcoRV 1.39
HindIII 1.54
pSK AtSHH Luc
6.59 Kb
NcoI 1.76
XbaI 1.86
Luc
EcoRI 2.16
35 S 3'
XbaI
BamHI
SphI
EcoRI
HindIII
SphI 2.94
BamHI
XbaI

Fig.7 ii.

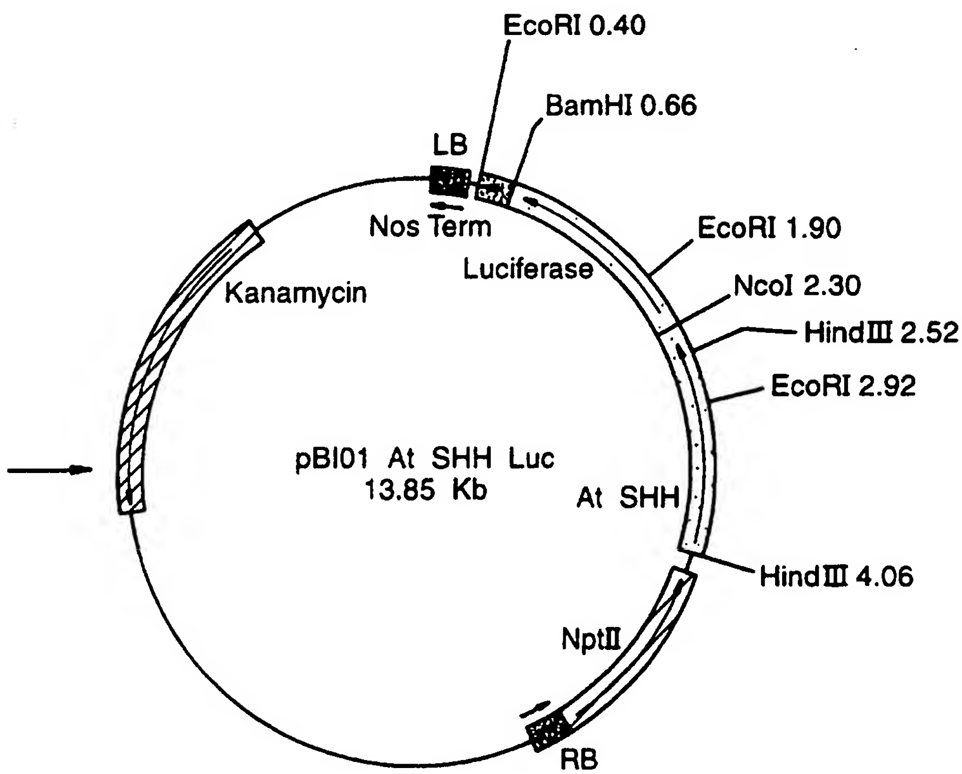
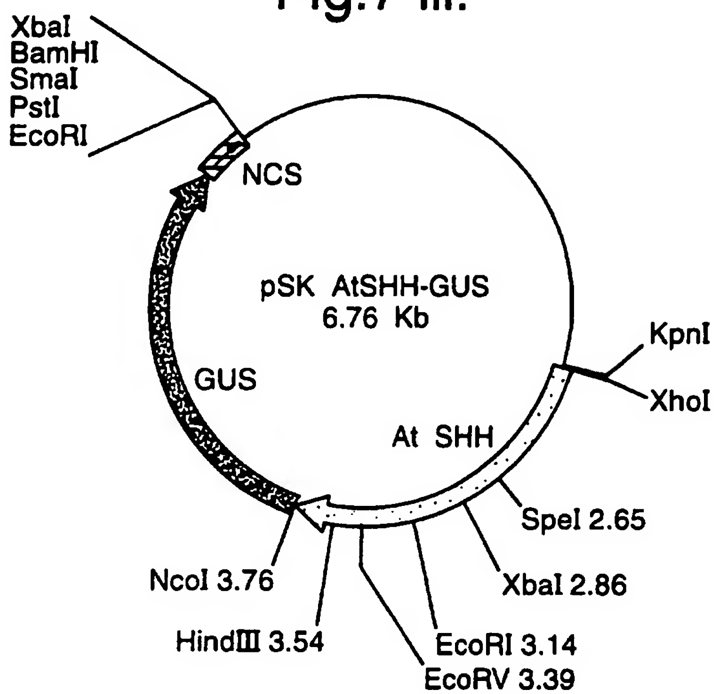

EcoRI 0.40
BamHI 0.66
LB
Nos Term
Luciferase
EcoRI 1.90
NcoI 2.30
HindIII 2.52
EcoRI 2.92
Kanamycin
pBI01 At SHH Luc
13.85 Kb
At SHH
HindIII 4.06
NptII
RB

Fig.7 iii.


XbaI
BamHI
SmaI
PstI
EcoRI
NCS
pSK AtSHH-GUS
6.76 Kb
KpnI
XhoI
GUS
At SHH
SpeI 2.65
XbaI 2.86
NcoI 3.76
HindIII 3.54
EcoRI 3.14
EcoRV 3.39

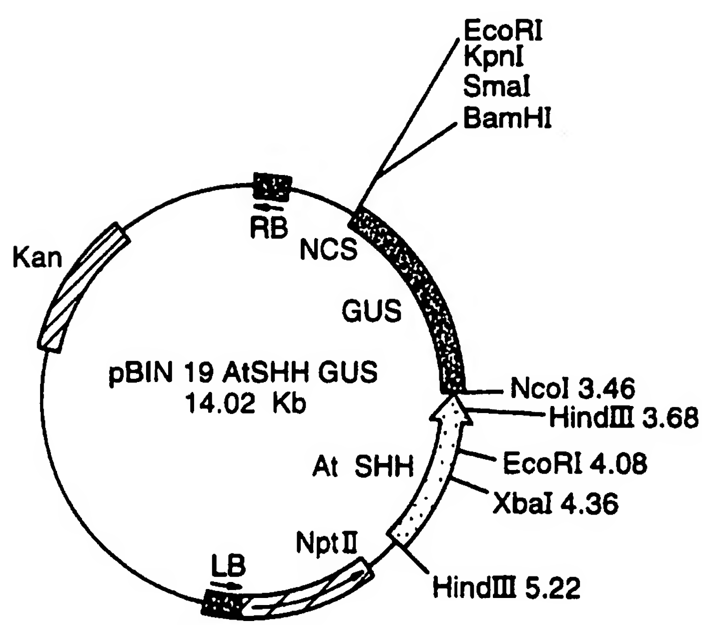
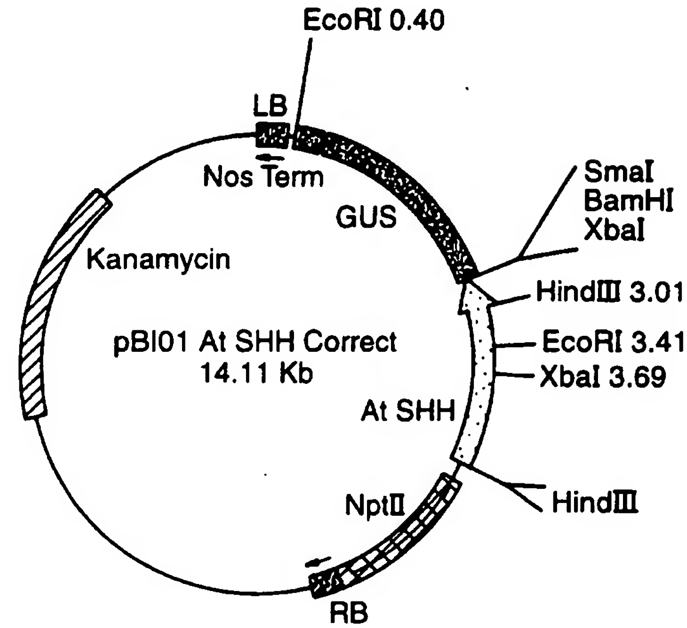

EcoRI
KpnI
SmaI
BamHI
RB
NCS
Kan
GUS
pBIN 19 AtSHH GUS
14.02 Kb
NcoI 3.46
HindIII 3.68
At SHH
EcoRI 4.08
XbaI 4.36
NptII
LB
HindIII 5.22

Fig.7 iv.


EcoRI 0.40
LB
Nos Term
GUS
SmaI
BamHI
XbaI
Kanamycin
HindIII 3.01
pBI01 At SHH Correct
14.11 Kb
EcoRI 3.41
XbaI 3.69
At SHH
NptII
HindIII
RB

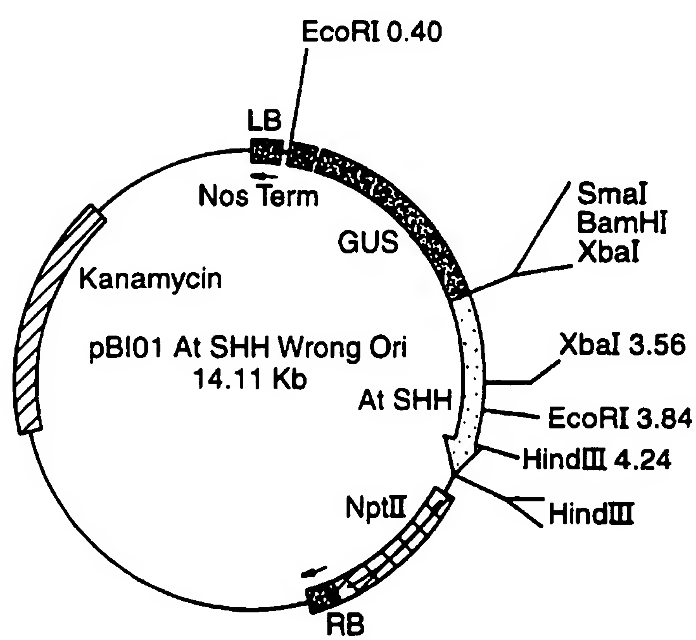

EcoRI 0.40
LB
Nos Term
GUS
SmaI
BamHI
XbaI
Kanamycin
pBI01 At SHH Wrong Ori
14.11 Kb
XbaI 3.56
At SHH
EcoRI 3.84
HindIII 4.24
NptII
HindIII
RB

Fig.8.

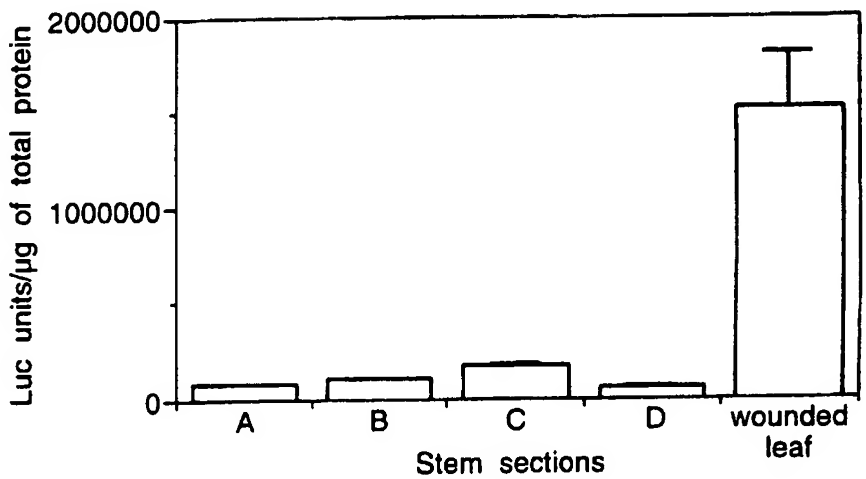

2000000
1000000
0
Luc units/µg of total protein
A
B
C
D
wounded leaf
Stem sections

Fig.9.

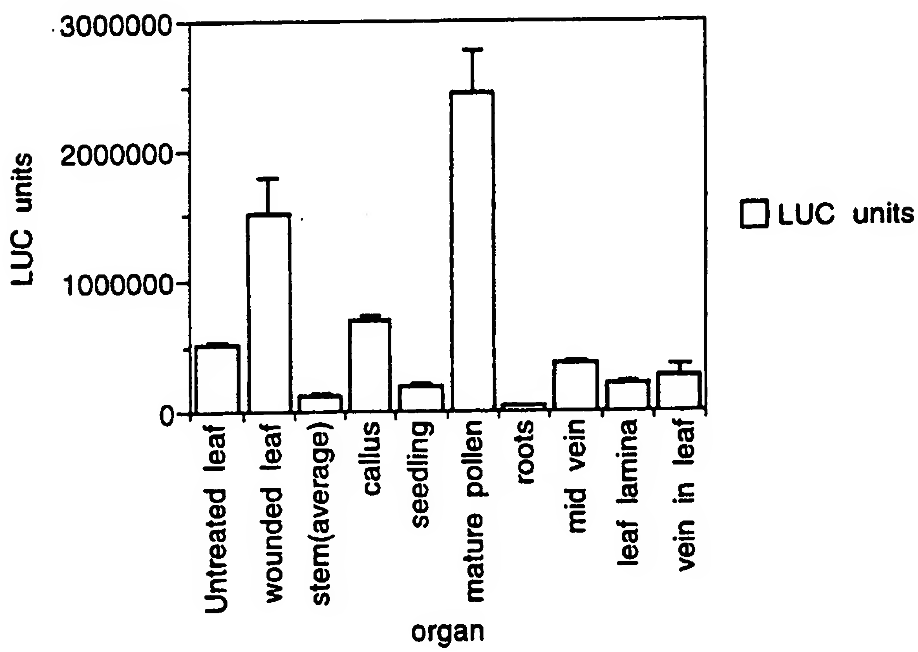

3000000
2000000
1000000
0
LUC units
Untreated leaf
wounded leaf
stem(average)
callus
seedling
mature pollen
roots
mid vein
leaf lamina
vein in leaf
organ
LUC units

Fig.10.

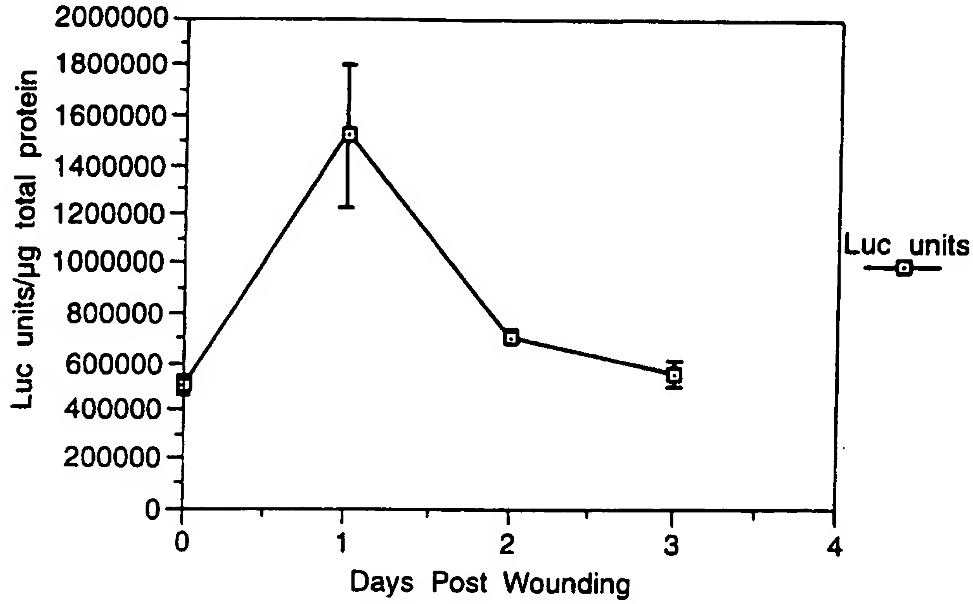

2000000
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
Luc units/µg total protein
0
1
2
3
4
Days Post Wounding
Luc units

Fig.11.

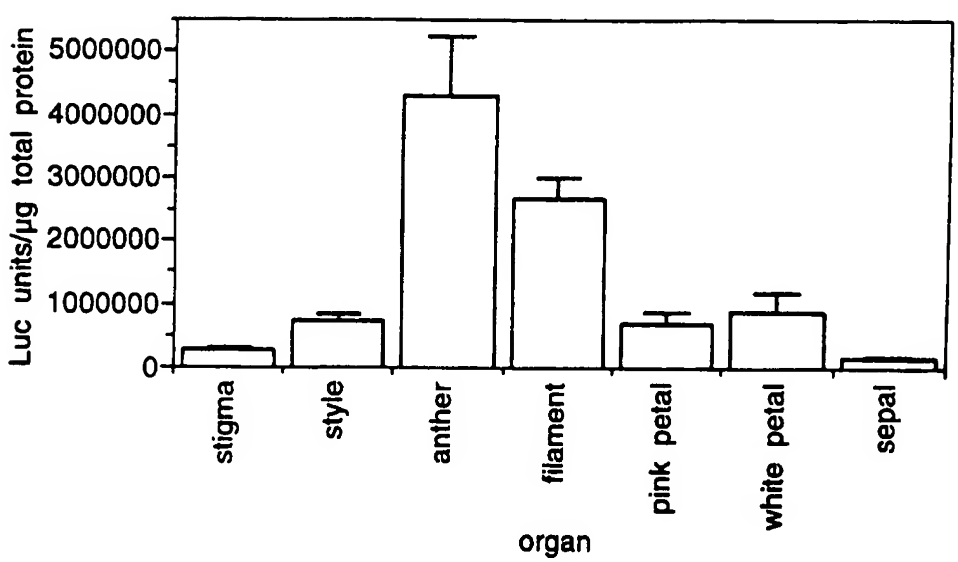

5000000
4000000
3000000
2000000
1000000
0
Luc units/µg total protein
stigma
style
anther
filament
pink petal
white petal
sepal
organ

Fig.12.

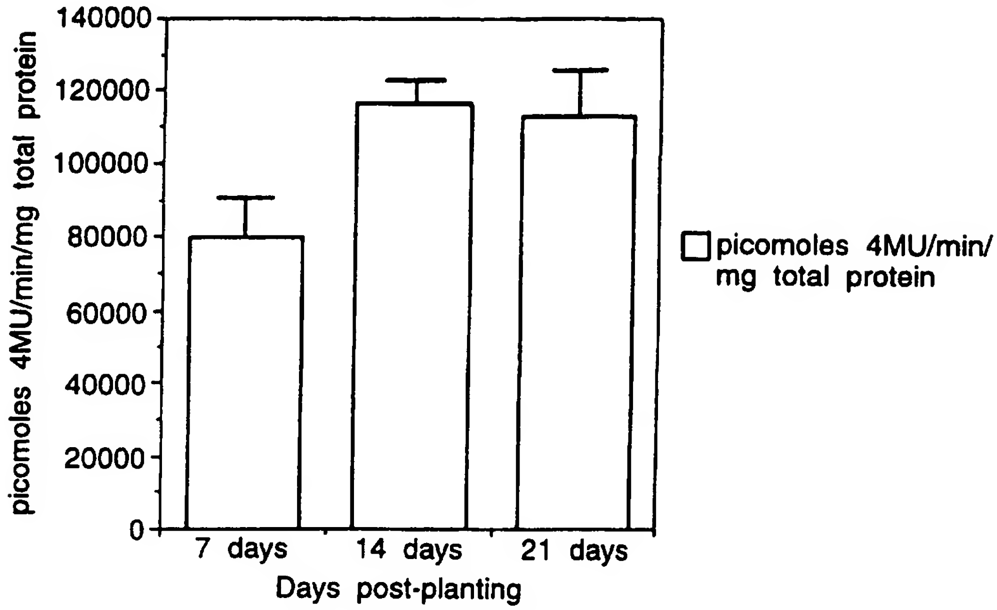
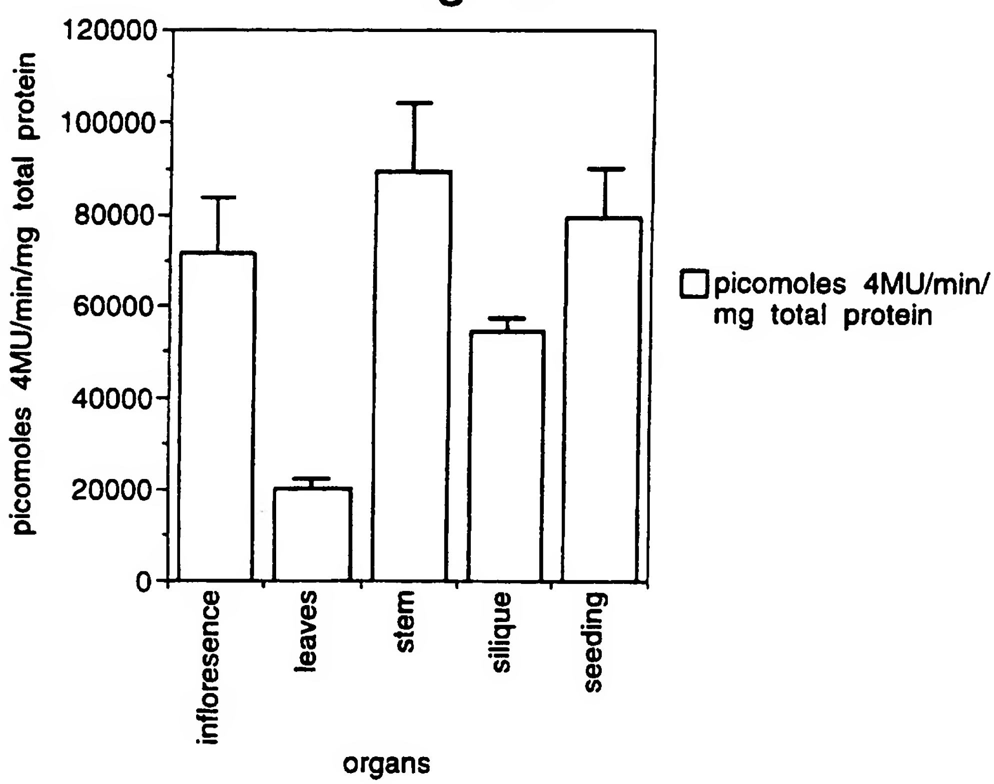

picomoles 4MU/min/mg total protein
140000
120000
100000
80000
60000
40000
20000
0
7 days
14 days
21 days
Days post-planting
picomoles 4MU/min/
mg total protein

Fig.13.


picomoles 4MU/min/mg total protein
120000
100000
80000
60000
40000
20000
0
infloresence
leaves
stem
silique
seeding
organs
picomoles 4MU/min/
mg total protein

INTERNATIONAL SEARCH REPORT

Intern   nal Application No
PCT/GB 96/00882

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/55 C12N5/10 A01H5/00 A01N65/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H A01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EUROPEAN JOURNAL OF BIOCHEMISTRY 229 (2). 1995. 575-582. , April 1995, XP000601433 MERTA A ET AL: "The gene and pseudogenes of rat S-adenosyl-L-homocysteine hydrolase." see the whole document ---	1,5,7
X	JOURNAL OF BACTERIOLOGY 176 (1). 1994. 61-69. , XP000601459 BUGGY J J ET AL: "Nucleotide sequence and characterization of the Rhodobacter capsulatus hvrB gene: HvrB is an activator of S-adenosyl-L- homocysteine hydrolase expression and is a membrane of the LysR family." see the whole document ---	1,5,7

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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1

Date of the actual completion of the international search	Date of mailing of the international search report
5 September 1996	13.09.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	PLANT SCIENCE (SHANNON) 113 (2). 1996. 167-174. , XP000600640 TANAKA H ET AL: "Inducible expression by plant hormones of S-adenosyl-L-homocysteine hydrolase gene from Nicotiana tabacum during early flower bud formation in vitro." see the whole document ---	1,5-11
X	ANNUAL MEETING OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY, SWANSEA, WALES, UK, APRIL 11-15, 1994. JOURNAL OF EXPERIMENTAL BOTANY 45 (SUPPL.). 1994. 12. , XP000600639 SKIPSEY M ET AL: "The cloning and characterisation of s-adenosyl-l-homocysteine hydrolase."	1,2,5-11
Y	see abstract P2.32 ---	12,13
Y	EP,A,0 492 536 (MAX PLANCK GESELLSCHAFT) 1 July 1992 see page 8, line 46 - page 9, line 32 ---	12,13
A	PLANT MOLECULAR BIOLOGY, vol. 21, 1993, pages 385-389, XP002012672 TREZZINI, G.F., ET AL.: "Isolation of putative defense-related genes from Arabidopsis thaliana and expression in fungal elicitor treated cells" ---	1-11
A	WO,A,93 19188 (MAX PLANCK GESELLSCHAFT) 30 September 1993 see the whole document ---	12,13
A	DATABASE WPI Section Ch, Week 9243 Derwent Publications Ltd., London, GB; Class C06, AN 92-354683 XP002012669 & JP,A,04 258 292 (JAPAN TOBACCO INC) , 14 September 1992 see abstract -----	1-13

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 96/00882

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0492536	01-07-92	DE-A- 4040954	25-06-92
		AU-B- 657929	30-03-95
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		JP-A- 6113856	26-04-94
WO-A-9319188	30-09-93	AU-B- 3751393	21-10-93
		CA-A- 2132323	30-09-93
		EP-A- 0631629	04-01-95
		JP-T- 7506485	20-07-95